The Diastatic Enzymes of Wheat Flour and Their Relation to Flour Strength

A THESIS SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF THE UNIVERSITY OF MINNESOTA

BY

LOUYS A. RUMSEY, B.S., M. S.

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

JUNE, 1922

CHICAGO, ILL.

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Research Fellowship Plan Under Which Fellows of the American Institute of Baking Have Been Enrolled as Graduate Students of the University of Minnesota.

The American Institute of Baking in the fall of 1920 detailed two research fellows in the chemistry of baking to work on suitable problems as graduate students of the University of Minnesota. These fellows were regularly registered in the Graduate School of the University. They pursued such courses as ordinarily constitute a study program of candidates for the doctorate in philosophy, majoring in the Division of Agricultural Biochemistry. Research problems were selected and outlined in conference with their advisors in this Division, actual work on the problems being pursued, by special agreement, chiefly in the laboratories of the American Institute of Baking. Theses based on this research were duly presented in partial fulfillment of the degree of Doctor of Philosophy, and accepted by committees of the Graduate School of the University. These theses are, by agreement with the graduate faculty of the Division of Agricultural Biochemistry, published by their respective authors as bulletins of the American Institute of Baking.

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THE DIASTATIC ENZYMES OF WHEAT FLOUR AND THEIR RELATION TO FLOUR STRENGTH

By Louys A. Rumsey

INTRODUCTION

The role which the enzymes play in the production of a good loaf of bread and their importance as contributing factors in the strength of flour is a matter of real concern to both the baker and the miller. After years of chemical and physical investigation of wheats and flours the cereal technologist is still confronted with the problem of finding some factor, or group of factors, the determination of which will furnish a sure index to the baking strength of a given flour. In spite of laboratory control in the blending and milling of wheats the wide variations in the ability of the corresponding flour to produce a "large, well piled loaf" (Humphries and Biffen, 1907.) still necessitates an actual baking test on each flour.

Gluten Content.—Because of the unique property of wheat proteins to form a tenacious, elastic framework of gluten for the retention of the aerating gas, attention was first turned to the amount and character of the gluten content. The numerous contributions of Osborne (1895 to 1907), Guthrie (1896), (1900), Fleurent (1896), Snyder (1899), Guess (1900), Humphries (1907-1910), Guthrie and Norris (1909), Wood and Hardy (1909), Bailey (1916), Blish (1916), Upson and Calvin (1916), Swanson and Tague (1917), Gortner and Doherty (1918), and others have led to the conclusion that in the proteins of the flour, and especially in the gluten, is to be found the most important basis for differences in baking value.

Protein Analyses.—On the other hand Fleurent (1901), Snyder (1901), Shutt (1905-1908), Norton (1906), Chamberlain (1906), Wood (1907), Thatcher (1907), Armstrong (1910), Ladd and Bailey (1910), Bailey (1913-1914), Blish (1916), and Martin (1920), have shown that percentage relationships from protein analyses would not suffice for the predetermination of flour strength. Neither does the accurate characterization of the proteins by Osborne and his co-workers apply directly to the problem of strength.

Colloids.—A more promising point of attack appeared to lie in the colloidal studies of the wheat gluten, the work on which is well summarized to 1918 by Gortner and Doherty. Physico-chemical studies of the gluten colloids (Gortner and Sharp, 1921.) involving viscosity measurements of the wheat flour proteins at different hydrogen ion concentrations, being conducted in the Division of Agricultural Bio-

chemistry, University of Minnesota, give promise of furnishing a most valuable index of strength for those flours in which strength is determined by gluten quality.

Climate.—The experience of the millers as well as the direct experimental evidence summarized by the reports of LeClerc (1909, 1910, 1912, 1914), Bailey (1913), and Thatcher (1913), have demonstrated that environment is the most important factor in the development of the wheat kernel. The biological differences in the relationships of various constituents are most marked when the same seed is grown under different climatic conditions. While changes of soil impose certain changes in the protein, ash, and carbohydrate content of the same wheats, the correlation is by no means as simple and direct as that between climate and "strength" of the wheat flour. Likewise different varieties and types of wheat respond most readily to climatic changes. The corresponding "strength" characteristics have consequently come to be regarded as typical of certain growing regions. We should expect the same changes to be manifested in the respective enzymes of the wheat kernel as carried over into the flour. For instance, a hard spring wheat, cut and shocked before it is biologically ripe, should be expected to show a different diastase activity than a softer wheat grown in the dry and semi-arid regions where the "dead ripe" grain is allowed to stand in the field under a hot sun and hot winds for days before cutting and threshing. Humphries (1910) came to the conclusion that such climatic factors did operate so as to change the diastatic activity of the flours from these wheats as well as their other strength characteristics.

Electrolytes.—The addition of various electrolytes as flour "improvers" and as yeast nutrients, bears a close relationship to the problem of gluten quality. As early as 1905 Fleurent began to investigate the effects of different acids and salts on gluten. Wood (1907), Shutt (1908), Wood and Hardy (1909), Humphries (1910), Willard and Swanson (1912), and Olson (1917), are among the earlier contributors to the study of electrolytes and their relation to strength. Numerous experiments with flour "improvers" and yeast stimulants have furnished a basis for the widespread use of various electrolytes on a commercial scale for the improvement of the baked loaf. The most of these investigations are included in the reports of Jessen-Hansen (1911), Bacon (1916), Kohman (1915), and (1916), Kohman, Hoffman, et al (1916), Hoffman (1917), Thomas (1917), Henderson, Fenn, and Cohn (1919), and Henderson, Cohn, Cathcart, Wachman and Fenn (1919). The real action of such added materials, for example on the gluten or on the enzymes, is but poorly understood as yet, and the whole subject needs further research.

Electrometric Methods.—The introduction of electrometric methods into the field of biological research has both facilitated and stimulated new methods of attack on the problem of flour strength. Determinations of the hydrogen ion concentration of flour, dough, and bread have helped to clear up some vexing questions, the most notable of which are: the control of undesirable organisms in bread, Cohn and Henderson (1918), Cohn, et al (1918), Morrison and Collatz (1921); the optimum acidity for proper fermentation, Jessen-Hansen (1911), Cohn, Cathcart and Henderson (1918); the optimum hydrogen ion concentration for enzyme actions, Sherman and Walker (1917), Sherman, Thomas and Baldwin (1919); and the lack of relationship between true and titrable acidity, Swanson and Tague (1919). Quite recently Bailey (1918), Bailey and Collatz (1921), and Bailey and Peterson (1921), have applied electrometric measurements successfully to the determination of electrolyte content of flours in their relation to flour grade. Collatz and Bailey (1921), have likewise been able to measure results of phytase activity by means of electrical conductivity measurements.

Gas Retention and Gas Production.—Wood in 1907, Humphries in his discussion of Baker and Hulton's paper on flour strength 1908, and Armstrong in 1910, all emphasized their opinions as to the importance of gas retention by a dough in relation to the gas production during fermentation. In 1916 Bailey proposed a method for comparative measurements of this ratio by means of an "expansimeter". Martin (1920) has published the results of comparative tests on the gas producing capacity, and gas retaining power of flours based on the fermentation method of Wood. He summarizes these two factors in flour strength by ascribing to a strong flour a **minimum gas-producing** capacity with a **high gas-retaining** capacity. Bailey and Weigley (1922) have just completed a report of some new studies on the retention of carbon dioxide in doughs in relation to gas production which lead to the interesting preposition that part of the ripening of the dough, and much of the spring in the oven is due to the carbon dioxide gas dissolved in the dough. Also their conclusions relative to the ratio of gas production to gas retention factors are in agreement with the hypothesis of Humphries, Armstrong, Biffen and others that the carbon dioxide production-rate is one of the important factors in flour strength.

Carbohydrate Content of Flour.—The intimate relationship between alcoholic fermentation and the proper aeration of the dough necessitated data on the carbohydrate content of wheat flour. But the analyses of Teller (1898), (1912), Stone (1896 and 1897), Brown, Morris, and Millar (1897), König (1898), Shutt (1907, 1908), Alway

and Hartzell (1909), Liebig (1909), Jago (1911), Thatcher (1913, 1915), Olson (1917), all verify the same conclusion, namely, that the small amounts of soluble carbohydrates naturally occurring in flour are insignificant in comparison to the amounts required for proper fermentation in the dough batch.

It should be borne in mind that there may be some correlation between the natural sugar content of the wheat and its relative state of biological ripeness. The investigations of Teller, Shutt, Thatcher, and Olsen, just referred to, would indicate the effect of climatic factors on the sugar content in flour milled from certain wheats.

Enzymes.—A natural corollary to the study of carbohydrate content is the investigation of those enzymes which are responsible for the production of carbohydrates available for yeast growth and fermentation, and those which alter and "condition" the gluten. Wood (1907), and Humphries (1907, 1910), Liebig (1909), recognized the greater importance of diastatic action than of sugar content in panary fermentation, and they reported measurements of the activity of such enzymes. These earlier experiments, along with other preliminary work on the enzymes of wheat flour bore evidence of their importance as contributory factors in the production of a good loaf. In addition to diastase, the other enzymes which have received the most study relative to their action in flour and dough are the proteases, the phytase, and catalase. The presence of a cytase, acting upon the outer surface or envelope of starch cellulose, has been postulated, but the evidence for the activity of this enzyme in flour is inconclusive. For the purpose of this report the activities of these different enzymes can best be considered along with the discussion of diastase.

Strength Probably Due to the Interaction of Several Factors.—It has become evident from the accumulated data resulting from years of investigation that no single analytical factor has yet been found which will suffice to predetermine the baking strength of a given flour. The baking test cannot as yet be replaced by any accurate means of predetermining baking value. It therefore becomes necessary to continue the studies of the various factors which go to make up this property of flour strength, not by themselves, but **relatively** and **collectively.**

Biochemical Method.—The wheat berry containing the plant embryo and associated food stored there for nourishment of the plantlet is produced by the plant for the propagation of its own species. The commercial flours produced by the roller milling process, and known as patent, straight, first and second clears, contain about 70 to 80 percent of the wheat berry, mostly of the food storage material, with

increasing amounts of the branny coverings and fragments of embryo, in the order named. Any differences in the laying down or "setting" of this storage material, or in the development of the embryonic elements, were imposed by the biological changes during growth, ripening, and storing of the wheat, and will, therefore, be reflected in the quality of the flour after milling. Studies on the progressive development of the wheat kernel by Teller (1898), Wymper (1909), Brenchley and Hall (1909), Thatcher (1913, 1915), Eckerson (1917); and those on the chemical changes in wheat under various conditions of storage or handling by Swanson (1916), Olson (1917), Bailey and Gujar (1918-1920), and Blish (1920), will justify our belief in the dependence of flour quality upon biological bases. The problem of flour strength must, therefore, be approached from the bio-chemical point of view: first, to gather all the available information concerning the character and properties of each individual biological factor contributing to flour strength; and second, to study these factors **relatively** and **collectively** in their many and labile relationships. The factors to be controlled in such an undertaking appear to be almost without limit, and, therefore, the task of a collective study of flour strength to be almost hopeless. Yet by judicious selection of constants and variables, a beginning is made which automatically leads to a better knowledge of the relationships of protein content, gluten quality as shown by viscosity and other colloidal properties, enzyme activity, carbohydrate content, gas retaining ability, yeast activity and fermetation, effect of shortening agents, acidity, buffer action of salts, chemical "improvers" and yeast stimulants, temperature, time, etc.

Purpose.—While this investigation was undertaken primarily to set aside and study in detail the diastatic enzymes of flours in their relation to flour strength, the plan also included the bringing together of as many controlled factors as possible to bear on a series of selected flours. The data published by earlier workers had been largely confined to the study of one or two factors on a limited number of flours, largely because of the enormous expenditure of time and energy required to make a more collective study. These flours cannot later be duplicated. And inasmuch as flours differ so widely among themselves it has been difficult for another worker to take up the problem and secure comparative results even when the same technique is employed. This applies even more strongly when attempts are made to correlate data by different workers on different factors. It was hoped to overcome this difficulty to some extent in the present work, first, by collecting fifteen samples of flour milled from authentic samples of wheat and representing various typical

wheat producing areas of North America; second, by the cooperative work of three investigators working on the same set of samples but from different points of attack; and third, by a complete record of all the available analytical data obtained on each flour. The cooperation of the American Institute of Baking research laboratories with the Division of Agricultural Biochemistry of the University of Minnesota made such a plan possible. The work on this series of flours has now extended over nearly two years. The present paper is the first report. Additional factors will be considered by the other collaborators in subsequent publications.

HISTORICAL

Payen and Perzos in 1833 proposed the term diastase to designate that active agent in malt which transformed starch to dextrins and some form of sugar. Jago (1911) further characterized as diastase that agent of malt which transforms starch or soluble starch to maltose. In the voluminous literature on the subject of amylase and diastase, the terms are sometimes used interchangeably. Later tendency is to distinguish between amyloclastic and saccharogenic action depending upon which activity is to be measured. Unfortunately this differentiation has not been as general as might be desired.

Definition of Diastase.—Leaving a consideration of the character of the two enzymes involved for later discussion, it should be stated here that the term diastase is restricted throughout this paper to mean "That enzyme or group of enzymes which through their successive or cumulative action produce maltose from the starch available."

It is impractical to consider in detail the great mass of literature on the subject of diastase in the malting and brewing industries except as it applies directly to the problem of better bread.

In 1907 T. B. Wood published the results of some studies on the the factors influencing the size and shape of the loaf. His paper marks the transition from the purely chemical analytic to a biological point of view. Although a sharp distinction was not drawn between the amount of sugars in the flour and those produced by diastatic action, he clearly recognized "that the sugar so formed, together with that originally present, forms the source from which the yeast makes the carbon dioxide it produces when the dough is fermented." This refers to the baking process then prevalent in England in which little or no added sugar was used in the dough batch, the fermentation depending almost entirely upon the sugars produced by diastatic action. To arrive at some measure of the sugars thus available for yeast fermentation, 20. grams of flour with 20 cc of water and 0.5

grams of yeast were incubated at 35°C, and the volume of carbon dioxide given off was measured over a period of hours. The resulting data led to the conclusion that: "Here too the rate of gas evolution and the size of the loaf run parallel, and it seems certain, therefore, that it is more particularly the gas given off in the later stages of dough fermentation that determines the size of the loaf. This being so the size of the loaf will depend, not so much on the sugar present in the flour as such, as on the diastatic capacity, which will cause continued sugar formation, and consequently continued gas evolution in the dough. Probably, therefore, measurement of the gas evolved in the later stages of the fermentation would give a more accurate test for the power of making a large loaf than the measurement which I have made of the total volume given off in 24 hours." Shutt (1907) in commenting on Wood's paper, expressed a belief, founded on analysis of sugars in flours, that diastatic activity rather than the sugar content in a flour is the determinative factor in loaf volume. His limit for reducing sugar is .62% and for non-reducing sugar 1.22%. A few months later there appeared the contributions of Baker and Hulton (1908) and Ford and Guthrie (1908). These two investigations, made independently of each other, were the first attempts at a systematic study of the enzymes of wheat flour in their relation to baking value. Ford (1904) had previously considered the estimation of diastatic power in malt flours, using soluble starch as a substrate. Many necessary precautions were pointed out concerning the preparation of pure soluble starch, and the effect of temperature, time, and acidity on the extraction of diastase, so that the results of this oft quoted article have an important bearing on the determination of diastatic activity in wheat flour.

Baker and Hulton's statement of the importance of diastase action in fermentation is in agreement with that of Wood as quoted above. They said "It is certain that some of the carbon dioxide concerned in the rise of bread, especially in the later stages of doughing and in the earlier period of baking, is formed from the fermentation of the maltose produced by the action of the diastase on the flour starch." They demonstrated the presence of diastatic action in a dough by extracting the sugars and preparing the osazones of glucose, and glucose and maltose, respectively, from two doughs made without yeast, one of which had the diastase inactivated by .02N NaOH, the other allowed to remain active for four hours at 40°C. But their measurements of the activity of flour extracts on soluble potato starch, although indicating its similarity to barley diastase (Baker, J. L., 1909), showed that large differences exist between this activity of extracts and the activity of the enzyme-con-

taining tissues when in the state of a dough, and it was recognized that this work on soluble starch, after the method of Lintner, gave no measure of liquifying enzymes. They found by measuring the amount of carbon dioxide produced during fermentation of a dough, as suggested by Wood, that there was a close correlation of carbon dioxide production to maltose resulting from diastatic activity, correcting for original sugars present. They also pointed out, however, that "a weak flour may have a diastatic power as high as or even higher than a strong flour."

The conclusions drawn from data on carbon dioxide production are of especial interest because with the exception of the few trials by Olson (1917), no results have been obtained for the liquifying agent in diastase of wheat flour. To quote from his conclusions: "The results indicate, we think, most conclusively that the low gas production of this flour (a weak flour) arises fron an inadequate supply of starch liquifying enzyme. We have already shown that the gas to diastase ratio is higher on the whole in strong flours, and it seemed probable, in view of the last experiment, that we might establish a connection between the strength and the relative amount of a starch liquifying enzyme in a flour."

The same phase of this problem was approached from a different angle by Ford and Guthrie (1908). Most of their efforts were directed toward obtaining complete extraction of the diastase from flour by applying the methods previously used for barley diastase. The effect of both added active proteoclastic enzymes, such as "papaien," and of various concentrations of added salts, such as sodium chloride, potassium chloride, and phosphates, were tried, resulting in the increase of diastatic activity in the extract. They state: "The amylase of wheat, like highly purified preparations of amylolytic enzymes from other sources does not exhibit its full hydrolytic activity except in presence of a certain salt concentration." This same principle of salt effect in the protection of diastatic action was made use of by Sherman and Walker (1917) and Sherman, Thomas and Baldwin (1919) in their studies on the amylases of animal, plant and fungus origin.

The data of Ford and Guthrie for the diastatic activity of their potassium chloride extracts from twelve different flours showed but slight differences in value as maltose per gram of flour. They carried autolytic measurements further than Baker and Hulton had done, but with the object of determining what effect the proteoclastic action would have on the diastase. After allowing the 1 : 25 flour water suspension to digest autolytically for different periods of time at 30°C. the diastase was extracted and its action on 2% soluble starch was

measured. The resulting data which are presented here as Table 1 shows the peculiar fact that such a procedure gives practically a constant diastatic activity regardless of time. The results of Thatcher and Koch which are discussed later, showed similar results with the same sort of treatment.

TABLE I.

"Autodigestion of Wheaten Flour"

(The digestion of soluble starch by diastase extracted from flour-water suspensions which had been allowed to digest autolytically for different periods of time): (Ford & Guthrie, 1908).

	Grams Maltose per 1 g. Flour	
	Flour No. 1	Flour No. 2
After 1 hour	2.87	11.83
After 3 hours	2.87	11.90
After 4 hours	2.80	11.69
After 5 hours	2.66	11.34
After 26 hours	2.52	12.74

One noteworthy suggestion in this contribution is that perhaps the degradation of the gluten in weaker flours by the proteases in some diastatic malt preparations is the reason for their failure to improve the quality of the loaf. Confirmation of this hypothesis still awaits further research on the proteases of wheat and malt flours. Preliminary investigations along this line in this laboratory point to the undoubted importance of proteases as factors in panary fermentation.

Baker and Hulton also had considered the proteoclastic enzymes of the flour, but expressed the opinion that these enzymes do not exert sufficient effect on the gluten during the baking period to be of serious consequence in the baking value of a flour. On the other hand, however, they point out that the proteoclastic enzyme of the yeast probably play a more important part in the modification of the gluten during fermentation. This fact was demonstrated by the presence of 2.7% of soluble nitrogen (as protein) in a fermented dough as compared to 1.9% in the same dough made without yeast. Liebig in 1909 reported the results of comparative sugar determinations made on wheat meal and on doughs made of the same meal. He found that maltose was steadily produced by the action of diastatic enzymes in a dough after standing fourteen hours at 30 to 40 degrees, and the reducing sugar content calculated as dextrose amounted to 4.6 percent. Doughs fermented two hours with yeast showed an excess of unfermented reducing sugar which Liebig concluded was due to the action of diastase.

There was a great deal of general interest shown by the chemists at that time in the diastatic and proteoclastic enzymes of wheat flour. Neumann and Salecker (1908), and Kohman (1909), demonstrated the improvement of bread by the addition of active dias-

tatic malt preparations. Humphries (1910) stated very clearly the position of diastase as a factor in flour strength. Armstrong (1910) likewise discussed its practical importance in the bakery but he doubted the possibility of correlating diastatic power with flour strength. Armstrong objected to Wood's (1907) measurements of gas production and diastatic power because "they were made under conditions very different from those which prevail in actual bakehouse practice."

Most of the earlier data in the literature, while significant, is of less value than its accompanying discussion. This is true chiefly because of the difficulty in controlling the conditions necessary for a study of the wheat flour enzymes.

Sherman, Kendall and Clark (1910), reviewed the various methods for use in the determination of diastatic power as a preliminary to a careful series of studies on the amylases of different origin. The method and the scale adopted by them depended upon the weight of maltose produced by the action of the diastatic preparation on soluble starch at 40°C for 30 minutes. Sherman's prediction (1910) that the determination of diastatic power would "soon become an important factor in the valuation of commercial American Malts," has become a fact. The registration of diastatic malt preparations for sale to the baking trade has shown the necessity for practical, uniform, standard methods of measurement. (American Institute of Baking, 1921.) The measurement of diastatic power as carried out at the present time among the control laboratories of this country show the most surprising variations in method and result. The Lintner method (1886), or one of its modifications, is still the basis for most of them.

The most important data in the literature to date concerning the autolytic diastatic activity in wheat flours is that of Swanson and Calvin (1913). They stated: "If flour itself possesses such large diastase activity that by digesting it with water at a suitable temperature, more than one fifth of its weight is transformed into soluble carbohydrates such as glucose and maltose, the conditions for such transformations deserve careful study." These authors allowed the diastase of the wheat flour to act autolytically on the wheat starch in water suspensions of various concentrations, measuring the amount of maltose produced. This procedure would appear to give an index of the activity to be expected in a dough. But no use has been made of this valuable data in the improvement of baking science. It is now impossible to duplicate the flours they used; nevertheless, the data can be considered as quite comparable to that obtained on other flours of similar quality, and so may be used on that basis.

The problem of diastase in the cereal grains was again attacked by Thatcher and Koch in 1914. Instead of applying auto-digestion as did Swanson and Calvin, they undertook to obtain extracts of constant diastatic power by digestion of the ground cereal with water at 0°C for two hours. This extract was then filtered in the cold, allowed to act on soluble starch for 30 minutes at 40°C, and the resulting maltose determined. The tables of results show some variation with time of extraction, which was due to the loss of diastatic power with a corresponding increase of sugars even at the low temperatures employed. Comparative results for one to five hour extractions could only be obtained because the time curve for diastatic action at 0°C rises so slowly that differences between one and three hours were within the experimental error of the methods employed (Fig. 2).

The probable error of a determination based on 30 minutes action at 40°C is large, because this point occurs on the steeper part of the curve, where differences of 1°C or 1. minute in time is considerable. The fact that no significant differences were observed between medium and fine grinding of the materials apparently eliminates at least one possible variant in the study of cereal diastase. Their results confirm the precautions necessary in the determination of malt diastase as pointed out by Kjeldahl, Ford, Brown and Glendening, and others, namely, there must be present so large an excess of starch that less than 40% is hydrolyzed to sugars.

Swanson, with Fitz and Dunton, published in 1916 the results of milling and baking tests on wheats which had been subjected to different methods of handling and storing. The effect of increased diastatic enzymes by the addition of small amounts of germinated wheat flour was to increase the "spring" in the oven and increase the volume of the loaf. Too large amounts, or flour from wheat which had germinated over too long a period, resulted in a weakened gluten, poor texture, and an inferior loaf of bread. More conclusive data was published the next year by Olson (1917), who made comparative baking tests on strongly diastatic flours milled from germinated wheat. No measurements were made on the relative saccharogenic powers of these flours, but their amyloclastic powers were compared by incubating the 1 : 20 flour-water mixture at 70°C. until the iodine test failed to show the characteristic blue color. The increase of amylase activity in the flour from germinated grain with a one centimeter epicotyl as compared to that from grain which had only started to germinate, was remarkable. Loaves baked from the germinated wheat flour showed increases in volume, but those containing too much amylase suffered in texture and character of crumb because

of too great liquifying action. This dextrinizing action supports Humphries' (1910) contention that added diastase in small quantity had the property of very perceptibly improving the flavor of a loaf, probably due to the correlation of increased dextrin content and the consequently improved conditions for the retention of moisture within the loaf. Olson showed further that the addition of varying small quantities of these diastatic flours to a normal flour did increase the water-holding capacity in direct proportion to the amount of germinated wheat flour added. In one case the volume of the loaves were increased as much as 49% without apparent impairment of the quality of bread, and in all cases there was an increase in volume, but the important fact in this connection depends upon the inherent "strength" of the normal flour used. The gluten of some weaker flours is of such poor quality that it cannot retain the extra gas produced through the agency of an added excess of diastase, and consequently the texture of the loaf suffers. It appears from those results that added diastase within certain limits, improves the baking quality of a flour, but the inherent strength of the flour governs the quantity of diastase which may be used. These conclusions agree with the work of Humphries, Kohman, Neumann and Salecker.

The data on the variations of the different forms of nitrogen compounds in the germinated wheat flours also lead one to suspect that perhaps the proteoclastic enzymes, in their degradation of the gluten, have had a more profound influence in the impairment of the grain and texture of the poorer loaves than the action of the diastase. Confirmation of this probability must await further research on the activity of proteoclastic enzymes and their effect in baking. The recent work of F. J. Martin (1920) is in effect a continuation of the investigations of Wood (1907). The volumes of gas given off by fermentation of 20. grams of flour with 55% water, 1.2% salt, and 1.0% yeast, over a period of 24 hours at 29°C were measured at different points of time. The volumes of the dough were in close relation to the gas production during the first periods of fermentation. But after a normal fermentation period of about three hours the relation was complicated by the reduction of the gas retaining power of the dough, due to a weakening of the gluten. There are several points in Martin's paper which are of significance in a study of enzymatic activities in flour. The variation in the volumes of gas produced by the different flours at any stage of fermentation was not great, and could not be correlated closely with final loaf volume. But in the case of a weak flour, one which showed a low gas-producing power, the deficiency of gas production in the later stages of fermentation could be rectified by the addition of diastatic enzymes, with a consequent increase in

volume of the loaf. These results are in agreement with those of Kohman and Olson. The analytical data on Martin's flours offer some interesting comparisons. They would appear to show the possibility of obtaining a constant for the ratios between water soluble proteins, gas retaining powers, and bakers' marks. The difference between his gliadin figures and the so-called "amended gliadin" point to a possible basis for the rough measurement of proteoclastic activity, since the water soluble protein increased with length of extraction period, at the expense of the alcohol soluble protein. In the second paper Martin (1920), demonstrates an increase of gas producing capacity, e. g., diastatic power, with the increasing percentage of wet and dry gluten as the source of the flour progressed from the center of the endosperm outward to the cortex. This data would appear to corroborate the opinion of Teichek (1904), that diastase, while largely concentrated in the germ, is also distributed throughout the endosperm and extends throughout the wheat berry. The studies of Whymper (1909), Mann (1915), and others, on the other hand point to the embryo as the only source of diastase in the grain. A further examination of these results, with analyses of both diastatic action and nitrogen partition might lend support to the postulate that the diastase of the wheat berry is associated with, or held by the protein body and functions normally only in their presence. Also that the peptizing action of the proteoclastic enzymes on the proteins has some bearing on the action of the diastase.

The difficulty of using the expansion volume of a dough as a measure of diastatic capacity is due to the weakening of the gluten after the second or third hour. "Curves plotted to show the relation between the amounts of gas generated and the volumes of the doughs were fairly regular for the first part of the experiment, but erratic for the latter part." (Martin (1920). The recent work of Bailey and Weigley (1922) afford a more complete summary of this relationship.

PRELIMINARY DISCUSSION

There are a number of factors which limit the production of maltose in a dough by the agency of diastase.

1. Liquifying and Saccharogenic Action. — Baker and Hulton (1908) believed that the diastase of grains contained both a liquifying and a saccharogenic part so that the amyloclastic power would become the limiting factor in maltose production. This idea is frequently expressed in the literature on diastase in bread making, but to date no satisfactory methods have been developed to accurately measure it. Nor has that phase of the problem been undertaken in this investigation for reasons which will appear further on in this discussion.

The presence of these two distinct activities in malt and wheat diastase have nevertheless been well established.

Without attempting to analyze the numerous but inconclusive data on the identity of the two distinct activities in diastase, it is sufficient to recognize their interpendence in the production of a fermentable sugar from natural starch, and summarize the later work on the subject. Sherman and Schlesinger (1913) made comparative measurements of the amyloclastic and saccharogenic power of the malt and pancreatic preparations. Their paper offers a comprehensive list of references on the distinction between the two kinds of activity, but no conclusions are drawn as to the identity of the separate enzymes. While their results on pancreatic amylase indicate a fairly constant relationship between the amyloclastic and saccharongenic activity of their pancreatic preparations, the same method of measurement failed for the malt preparations, the amount of starch liquified apparently being less than the maltose produced therefrom. The explanation suggested to account for the observed discrepancy was: That these two activities are characterized by different conditions of optima for their respective rates, i. e., of temperature, acidity, and salt concentration. Sherman and Thomas (1915) supplied further evidence in support of that explanation by their measurements of the optima for amyloclastic and saccharongenic actions. Whether this explanation be the correct one, and the two distinct activities be due to separate, conjunctive enzymes; or whether the special properties of organic colloidal catalysts can perform the two degrees of hydrolytic power under the influence of changing conditions in media; it should be possible to more accurately characterize the amyloclastic and saccharogenic parts. To mention only a few of the possibilities, it would appear that the researches of Sorenson (1917), Robertson (1920), Meutscheller (1920), and Warden (1921), in the application of physical and electro chemistry to biological problems, furnish many methods which might be applicable to the separation and identification of these two enzymes if they exist as such, or to the establishment of the identity of the catalyst responsible for the different degrees of hydrolysis. The remarkable results of Warden add another link in the chain of accumulating evidence for the hypothesis that enzymic activity is but another manifestation of the catalytic process functioning by virtue of those surface forces which are active in colloidal particles. Such studies are obviously beyond the compass of this set of experiments and pertain more to the theory of enzyme action.

In the methods of Sherman and Schlesinger noted above, soluble (gelatinized) starch was again used for the measurement of both amyloclastic and saccharogenic power, and therefore cannot be

applied directly to the question of amyloclastic activity as the limiting factor in diastatic action in the dough.

2. Resistance of Different Starches to Diastatic Action. It is only the final production of maltose, resulting from the diastatic activity of a given flour, which is of consequence to the baker, and the various limiting factors, including the amyloclastic activity and the resistance of that particular starch, are summarized in that final result. Yet when a method is applied also to the measurement of diastatic powers of malt preparations, which are intended to act on the natural starches, the question of the resistance of the natural wheat starches becomes of first importance, both from the standpoint of the final product, and for the selection of a standard substrate.

There appears to be a difference in the condition of the starch in the various kinds of flour. The difference was noticed by us in working with suspensions of the flour samples under investigation. Whymper (1909) by microphoto examination of starch, showed differences in the resistance of different starch granules in flour to diastase. Simpson (1910) "has shown that under certain conditions a small proportion of flour converted into sugar a quantity of ungelatinized starch equal to 8% of the weight of the flour, but that under identical conditions the same quantity of the same flour converted a quantity equal to 400% of its own weight into sugar when gelatinized starch was used." Stone (1897), was of the opinion that there were differences in the action of enzymes on starches of different origin, but Ford (1904), confirming O'Sullivan's previous observation (1904), concluded that there is practically no difference in the action of diastase on starches even of different origin under comparable conditions of acidity and temperature. Their data, however, was obtained on modified, or "soluble" starch, and so may not be applied to the question of the biological differences in natural wheat starch. Sherman, Walker, and Caldwell (1919) also reached the conclusion that there was little or no difference between the resistance of different starches to the same diastase preparation, but their starches had likewise been boiled. The important work of Reichert (1916), and of Dox and Roark (1917), would lead one to expect a variation in the resistance of starches of different origin to the action of enzymes. Stakman (1918) and Leach (1919) have pointed out the significance of resistance to parasitism which is shown by different varieties of wheat. Considering the penetration of parasites into the host as the result of enzymatic action, the variable resistance of different biological conditions to enzymes may well be expected to show in the starch granules as well as in other plant tissues. The recent articles by Samec and his co-workers (1921) present some

extremely interesting possibilities in the chemical, physical, and electrometric differentiation between starches of different biological origin. But the susceptibility of biologically different samples of wheat starch to diastatic activity is but one of the controlling factors, and like the other factors, is reflected in the resulting degree of diastatic power.

3. Difference Between Autolytic and Extracted Enzyme Activity. In the wheat berry the diastase is laid down by the plant for the purpose of transforming the starch granules of the endosperm into soluble sugar available for assimilation and growth of the young seedling. Whether or not in the economy of the seed there is a separate preparatory or liquifying enzyme, a cytase which prepares the starch granule such as suggested by Armstrong (1910), and makes it susceptible to the hydrolytic action of the diastase (Wallerstein 1917, and others), the result is the same. At any rate during panary fermentation, the diastase of the flour, and likewise any diastase added to the dough in the form of malt flour, malt extract, or other diastatic preparation, must produce maltose from the wheat starch **as it exists in the flour,** unless other forms of starch be added to the dough.

The results of Ford and Guthrie (1908), have indicated that the greater enzymic activity was obtained by auto-digestion of malt, and by addition of salts and proteoclastic enzymes, those of Baker and Hulton (1908) that flour extracts do not furnish a true measure of the diastase present in the flour; those of Sherman and Baker (1916), that purified malt extracts show different activities on different forms of starch substrate (prepared), and those of Swanson and Calvin (1913) have demonstrated the value of the autolytic method for flours. Yet the methods in general use at present for the measurement of diastatic power all depend upon the action on "soluble" starch. In other words, an **artificial and by no means standard substrate, whose colloidal characteristics have been profoundly altered, is the starting point for an arbitrary procedure, the result of which often has no bearing on the activity intended to be measured.** It was mentioned above that the same diastase acting on soluble or gelatinized starch may produce several hundred times as much maltose as when allowed to act on raw or unbroken starch kernels. **It will be demonstrated later, (pp. 72, table XXII) that this is a very real source of error in the determination of diastatic power of different diastase preparations.** Thatcher and Koch also found that extracts prepared by their method gave lower diastatic values than were obtained in equal aliquots of unfiltered extracts from the same flour. A little later "attempts to apply the method in a comparative study of the diastatic activity of

wheat flour of different grades and various processes of manufacture gave the surprising result that extracts of approximately uniform diastatic qualities were obtained from flours of widely varying character and baking qualities."* Still more recently R. W. Thatcher and Cornelia Kennedy (1917) obtained some valuable data bearing on the loss of diastatic activity in flour extracts by various methods of treatment.** One series of flour samples were subjected to auto-digestion with water (1:4) for 1 hour at 0°, and the filtered extract allowed to act on soluble starch at different temperatures, as in the proposed method of Thatcher and Koch (1914). The following table shows the surprising difference in result:

TABLE II.

The difference in diastatic activity between auto-digestion and the action of extracted diastase on soluble starch.

Temperature of Action on Starch	Cuprous Oxide Produced by Diastase from .5 Grams of Flour: Auto-digestion of Flour-water (1:4) 1. hour Grams	Extracted 1 hr. @ 0°C Filtered Extract on Soluble Starch 1 hr. Grams
40°	.02390	.08485
50°	.05497	.08125
62°	.15296	.08050

It was likewise found that the unfiltered extract acting on soluble starch at 40, 50 and 62°C showed 1.673 g. Cu. per .5 g. flour as compared to only .4302 g. Cu. per .5 g. flour for the filtered extract; **a loss of about 74% of its activity through filtration.** An examination of the residue on the filter would indicate that the tenacious glutinous mass had adsorbed some of the enzyme, or else had held back an activator. This residue was apparently not tested directly for its activity. But samples of gluten were washed out in the usual manner and were kneaded with distilled water until no more starch could be removed. After dispersion in N/200 lactic acid their diastic activity on soluble starch was compared with an extract, both filtered and unfiltered. The results are given in Table III.

TABLE III.

The relative diastatic activity of water extracts and of gluten from the same flour.

1. 0.895 g. Cu. per .5 g. flour (Filtered extract on soluble starch)
2. 3.200 g. Cu. per .5 g. flour (Unfiltered extract on soluble starch)
3. 0.818 g. Cu. per .5 g. flour (Gluten dispersed with N/200 lactic)

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*(Geo. P. Koch's unpublished results in a thesis presented to the Faculty of the Graduate school of the University of Minnesota, in partial fulfilment of the requirements for the degree of Master of Science).

**(Grateful acknowledgment is hereby made to these authors for their permission to publish the results of their experimental data in this connection).

It would thus appear that the gluten had retained nearly half of the diastatic activity. Unfortunately there is no data available whereby a correction can be applied for a possible activating influence of the N/200 lactic acid. This absorption of enzyme by gluten was further examined by extracting 4. g. flour with 100 cc. H_2O, (in which were dissolved .006 g. takadiastase) for 1 hour @ 0°. Part of the extract was filtered and compared to the unfiltered portion in its action on soluble starch. The unfiltered extract produced 6.725 g. Cu. as against only 3.215 g. Cu. for the filtered extract. Baker and Hulton have found and recorded that some diastatic activity was shown by the glutens thus washed out of wheat flour.

In the course of these experiments of Thatcher and Kennedy it was desired to ascertain whether only maltose was produced by diastasis. The increase in Fehling reduction by inversion of the solution after diastatic action had been stopped was measured.

TABLE IV.

The relative reduction of Fehling solution before and after inversion of the sugars produced by diastatic action.

	Before Inversion grams cu.	After Inversion grams cu.
I.	.02208	.04416
II.	.03940	.06760
III.	.02784	.05376
IV.	.03264	.06192

In spite of the evidence of Sherman and Plunnett (1916), for the production of small amounts of glucose in addition to maltose by malt amylase, the fact that maltose is the only sugar produced by diastase in sufficient quantity to be of any significance in panary fermentation has been recently confirmed in this laboratory. (Collatz, 1922).

One peculiarity of the results obtained by Thatcher and Kennedy, however, still remains to be explained. The diastase activity in flour suspensions has been shown by Swanson and Calvin, and confirmed here, to be extremely sensitive to temperature, the curve appearing autocatalytic in character up to nearly 60°C. Yet the activity of the **filtered** extracts of Thatcher and Kennedy showed the same value whether acting on soluble starch at 40°, at 50, or at 62°C, while the unfiltered extract showed a temperature effect. There are obviously several possible explanations: (1) The filtration may remove some substance which functions as an activator (Thatcher, 1921); (2) The proteins of the flour may absorb some essential factor in the diastatic action, and; (3) the contributing, perhaps controlling, effect of the phosphates and other buffer salts present in the flour (Bailey and Collatz 1921, and Bailey and Peterson, 1921), do not function under those conditions of extraction and measurement. Neither of

these explanations seem to be sufficient. The enzymes are undoubtedly colloidal in character. Their hydrolytic activity, involved as it is in the complex properties of colloidal material such as surface action, adsorption, dispersion, etc., is, therefore, extremely sensitive to any changes in the electro-chemical and physical nature of the media in which they are acting.

It has been found that the starch as washed out of flour carries considerable diastase, the gluten retaining by far the greater portion Removal of the protein matter from the starch granules by dispersion and repeated washing effects the removal of the diastase, indicating that the diastase is neither adsorbed to nor associated with the starch. K. Mohs (1920, 1921), has presented interesting expositions on the colloidal theory of diastatic activity and though probably carried further than experimental facts warrant, they are extremely suggestive for experimental verification. It appears certain, in short, that extraction of diastase from biological material, with subsequent filtration and hydrolysis of modified starch by the filtrate, will not afford an accurate conception of the true activity as it exists **au naturel. Therefore, any practical method for the measurement of the value of diastatic enzymes in baking must be based upon conditions as they exist in the dough.** The increasing general use of diastatic preparations in baking practice for the improvement of bread makes it imperative that a new procedure be developed. It must give a measure of the ability of that diastatic preparation to produce sugars which can be utilized by the yeast for growth and carbon dioxide production.

EXPERIMENTAL

A survey of the literature on diastatic activity has shown the unquestionable importance of these enzymes in panary fermentation. The fact that the bakers in America are estimated to use something over thirty million pounds of malt per year, a large part of which is diastatic in character, with a probable valuation of over two and a half million dollars, is sufficient evidence of the economic interest in the problem. The relative importance of this activity as a factor in the composite strength of wheat flours has not been settled. The interest of the baker in this question has steadily grown until a satisfactory answer should contribute materially to baking science. Consequently the general use of diastatic preparations for the improvement in volume and flavor of bread, and the numerous but inconclusive contributions found in the literature on the effects of diastase in panary fermentation, have all served to demonstrate the great desirability of further research on the diastatic enzymes of wheat flour in their relation to flour strength.

The Materials. To obtain data which would furnish the basis for conclusive evidence as to the role taken by diastase in dough fermentation, it was considered necessary to obtain samples of wheat flours of widely varying characteristics. Samples of flour, of different grades and baking strengths were accordingly obtained from eight of the typical bread-flour wheat producing areas of North America. These districts include the Northern Great Plains area, the Washington Walla Walla, the Saskatchewan and Alberta Canadian, the Kansas, the Utah irrigated, the Montana Dry Farming and the Ohio wheat areas. The Sitka, Alaska mills were not yet completed and the Alaskan wheat sample obtained was too small to furnish sufficient standard flour for comparison with the other samples. The most of these samples of flour, in fifty pound lots, were obtained direct from the commercial mill in which they were ground. In the case of samples Nos. 1012, 1013, and 1014 the wheat was obtained from Fergus county, Montana, and was selected to represent the average wheat as raised in the district. It was shipped direct to Fargo, N. D. and there reduced to flour at the experimental mill operated by the Agricultural Experiment Station of North Dakota. In all cases samples of the wheat from which the flour was milled were kept for further check and examination. Thus the flours studied in reference to their diastatic activities are known to be representative of the wheat growing districts from whence they came. It should be remarked, however, that three of the samples, number 1001, 1002 and 1009 are milled from blended wheats. Numbers 1001 and 1002 are patent and 1st clear respectively, milled from a regular "mill mix" and are typical of the flours generally milled in that locality. Number 1009 should receive special mention because of its relation to the other flours. It is a well known commercial brand of high grade patent flour, milled from a two-wheat blend in the Northwest, and was selected because of its superior quality. The composite of its "strength" characteristics, as well as its combination of qualities as shown in the baked loaf, gave it the highest baking value of any of the flours available. Consequently this sample was used as a standard against which to compare all others.

The objection might be raised that these samples, numbering fourteen in all, do not furnish sufficient bases of seasonal and climatic variation for a complete characterization of enzymatic activity in relation to strength. Yet it is believed that sufficiently characteristic data has been obtained to justify certain conclusions and to furnish the foundation for further investigation into the behavior of these biological catalysts of wheat flour.

A history and description of the flour samples follows:

TABLE V.

History and Description of Flour Samples.

Sample No.	Locality (Where Grown)	Wheat Type	Wheat Class	Flour Grade
1001	Reno County Central Kansas	Turkey Red	Hard Red Winter	Patent
1002	Reno County Central Kansas	Turkey Red	Hard Red Winter	1st Clear
1003	Washington Walla Walla	Little Club	White Club	Straight
1004	Red River Valley North Dakota	Marquis and Bluestem	Hard Red Spring	2d Clear
1005	Red River Valley North Dakota	Marquis and Bluestem	Hard Red Spring	Patent
1006	Canada Southern Alberta	Marquis	Selected Hard Red Spring	Patent
1007	Canada Southern Alberta	Marquis	Selected Hard Red Spring	1st Clear
1008	Canada, Saskat. Valley, Saskatoon	Marquis	Hard Red Spring	Patent
1009	Red River Valley	Mostly Marquis	Hard Red Spring	Patent
1010	Utah Irrigated Valley	Like Sonora California Wheats		Straight
1011	Ohio Williams County	Resembles Fultzo-Mediterranean	Soft Red Winter	Long Patent
1012	Montana, Judith Basin, Dry Farming	Common Turkey Red	No. 2 Hard Red Winter	Patent
1013	Montana. Judith Basin, Dry Farming	Common Turkey Red	No. 2 Hard Red Winter	1st Clear
1014	Montana, Judith Basin, Dry Farming	Common Turkey Red	No. 2 Hard Red Winter	2d Clear

Further notations on the special characteristics of the individual samples will be recorded in connection with the experimental data.

In order to obtain a satisfactory conception of the relative baking strength of these flours it was first necessary to make preliminary baking tests. These were carried out by the baking expert of the American Institute of Baking, and under the author's constant surveillance and supervision. This baker had had wide experience with all types of flours in various parts of the country, and so was especially well fitted to bake and judge these loaves. The same individual baked all of the flours, in groups of four, repeating the standard and one of the other flours for comparison with each succeeding day's bake. The method used was that developed for a standard baking practice by this Institute for its service and research departments. The purpose of this standard baking test was to produce a loaf under carefully controlled and duplicable conditions which should approach as nearly as

possible to those obtaining in the average American bake shop. It is recognized, of course, that conditions of fermentation in a small dough batch must of necessity differ somewhat from those in a thousand pound dough. Nevertheless, it has been found that this difference, due to what the baker calls "mass action," can be largely compensated for by a proper increase in the amount of yeast and a proportionate allowance in temperature. Experience has shown that the behavior of a particular flour, as represented by the fermentation, and by the "score" of the finished loaf, can generally be taken by the experienced baker as a measure of the baking quality of that flour when subjected to the conditions of quantity production.

Baking Test. A description of the apparatus used in the baking tests will first be given so as to facilitate the discussion of the procedure. The service laboratory was equipped with a ten loaf, electrically heated Despatch bake oven, equipped with mercury and recording thermometers, and piped for low pressure steam. The fermentation box, 72x24x13 inches, and proofing cabinet, 58x22x13 inches, inside measurements, were electrically heated, with thermostatic control, and the proofing cabinet was also piped with low pressure steam for controlling the humidity. The one pound pans in which all the loaves in this series were baked have the following dimensions: Top, eight and five-eighths by four and five-sixteenths inches; bottom, eight and one-eighth by three and three-fourths; heignt, two and one half inches. A small Hobart three speed mixer fitted with a two pound bowl, was used for the mixing of all doughs.

Formula. The standard formula, in terms of a one pound loaf dough, is as follows:

	Grams	Per Cent
Flour	325.0	100.0
Water	179.0*	55.0
Sugar	10.0	3.0
Yeast	8.0	2.5
Salt	5.0	1.5
Lard	6.5	2.0

History and Description of Flour Samples.

*The amount of water added depends upon the absorption of the flour employed. The "absorption" was determined in the conventional manner, by doughing up 100 grams of the flour and recording the number of cubic centimeters of water required to produce a dough of the proper consistency. It is a well known fact that the "absorption" as determined in this manner must often be changed, depending upon whether the dough stiffens, or slackens during the course of fermentation. The absorption values as recorded in the subsequent data are those found by actual fermentation to be the most desirable for the proper fermentation of each particular flour. This is a departure from the custom which has usually been followed in comparative bake tests as recorded in the scientific literature, but in accordance with commercial practice the baker was instructed to modify the absorptions and fermentation times in such a way as to produce the best possible loaf, i. e. to show the greatest strength of the flour without the use of any additional ingredients.

The Baking Procedure.—Flour samples of 650 grams each (required for a two-loaf dough) were weighed out into ten inch mixing bowls and set in the fermentation cabinet over night at 27° centigrade. 200 grams of sugar and 100 grams of salt were weighed out together, dissolved in water, and made up to a volume of two liters. The lard was weighed out in 13 gram portions for each dough. The yeast was a part of the supply delivered fresh each morning for use in the baking school, and showed unusual uniformity in fermenting abiltiy. The yeast was cut from the center of a one pound cake, and 160 grams of this were weighed out thirty minutes before mixing the doughs. This yeast was suspended in water in a one liter flask at a temperature of 27 degrees. By the use of these solutions, 200 cc of sugar-salt solution furnished the required 2.5% of sugar and 1.5% of salt for each two loaf dough, while 100 cc of the yeast suspension contained 16 grams of compressed yeast.

It has been previously found that the 20. grams sugar, 10. grams salt, and 16. grams of yeast for each dough displaced just 21.6 cc of water. Therefore it was necessary to take this extra volume into account and add it to that volume of liquid as calculated from the absorption. For example, flour 1008 with an absorption of 60 should require $650 \times 60 = 390.$ cc of water, so in addition to 200 cc of sugar-salt solution and 100 cc yeast suspension, there would be required $90 + 21.6 = 111.6$ cc more water. The sugar-salt solution and the yeast suspension were both brought to approximately 27°, and the extra volume of water could be warmed or cooled as a convenient control for the temperature of the dough, which was always brought out of the mixer at an even 27°C. A half degree rise in temperature was allowed for each minute of mixing. To mix, the flour was transferred to the bowl of the machine mixer, 200 cc of sugar solution, and 100 cc of the well shaken yeast suspension were then added from rapid flowing pipetts, and the mixer started on the lowest speed. As soon as the flour had all been taken up in the dough the lard was added and mixing continued at second speed to the end of the second minute. The dough was then cut down from the revolving arm and mixed with the mixing arm turning at high speed for another minute. In this same manner each dough received the same thorough mixing of approximately the same number of revolutions, and of three minutes duration. Each dough was then accurately weighed, set in lightly greased bowls fitted with large clock glasses, and placed in the fermentation cabinet at 27°C (80.6°F). The average total fermentation time was five and one-half hours from mixing to baking, from the time of the first "punch." The dough was considered to be ready for the first working, variously termed "turning," "cutting over," "knead-

ing," "knocking down," or "punching," by the appearance of the dough surface when indented by the finger. If the outer edges of the indentation, instead of filling in again, should show a tendency to sag down, after a moment, the dough was considered ready for the first punch. This consisted of removing the dough from the bowl, kneading it lightly five or six times to expel most of the gas, and setting again. Considering this as 60% of the total fermentation time, the remaining period was divided into approximately 28% and 12% of the total time, the second punch following the first in about 50 to 60 minutes, and the dough going to the bench for rounding after a third punch about 25 to 30 minutes later.

The method of handling the dough from the mixing to the oven can best be illustrated by an example. Time mixed 9:20 a. m.—ready for first punch at 11:40 a. m.; from mix to first punch 140 minutes. Taking that as 60% of total time, the total fermentation period should be 233 minutes. Taking 28% of 233, or 65 mintues, the second punch would come at 12:45 p. m., and the third at 1:13 p. m. The third punch is in reality a rounding up of the dough, corresponding to the machine rounder in the modern bakery. After being rounded up the doughs were allowed to stand on the bench for fifteen minutes, then moulded into loaves.

In these test bakes the two loaves were carried through as one dough to facilitate handling and temperature control. The doughs were weighed and their temperature recorded at each punch. When taken to the bench for rounding, this dough was divided into two equal halves, and each half rounded up separately. In the first test bake the rounded doughs were moulded into loaves by hand, but in the second and third bakes uniformity of grain and texture was obtained by running them all through a Thomson machine moulder.

The loaves were then panned in separate pans and placed in the proofing cabinet where they remained at a temperature of 32.5°C, in an atmosphere nearly saturated with moisture, until ready to go into the oven. The duration of the proof was usually 55 to 60 minutes, depending somewhat on the flour, and the height to which the doughs were allowed to rise before baking was that which was found by experience to give the best appearing loaf with this type and size of pan.

When the loaves were ready for the oven, the steam was turned on to furnish a moist heat and delay crusting, and allowed to remain on for the first three minutes the loaves were in the oven. The temperature of the oven was so adjusted that it registered 435°F at this point in the baking. Twenty to twenty-five minutes' baking was sufficient to produce a well baked-out loaf with a deep golden-brown colored crust.

The loaves were weighed directly out of the oven, again after one hour, and a third time at the end of eighteen hours. The volume of the loaves was taken at this time with the Central Scientific Company's volume machine, using mustard seed, the volumes recorded having been repeatedly checked up and corrected by displacements in water. The loaves were scored and "placed" as to baking value, the resulting relative position being the result of independent judging by three experienced practical bakers. The numerical baking value assigned to each flour is the average of all the different scorings of three trial bakes, consisting of two loaves for each flour in each bake. The first series of baking tests was made in December, 1920. The baking on each loaf was repeated two or three times with slight variation in fermentation time and absorption as indicated by the possible improvement of the loaf. The second test bake was made the middle of March, 1921 in order to properly classify some new samples just received. The order of baking value and relative strength of flours had not altered appreciably over that three months' period. The third baking test was completed in November, 1921. Only the data for this last series need be given here, since the relative position and score of the baked loaves remained the same over the six months' period. And though the flour had aged somewhat, as shown by the natural pH of the water extract, the slight change in "absorption" and slightly improved baking characteristics, the final bakings represent very well the best "strength" characteristics of each flour.

Table VI is a record of the comparative baking tests; the final score being a summary of points according to the American Institute of Baking standard (1922).

TABLE VI.

Record of Comparative Baking Tests.

Flour Sample	Absorption	Times: Fermentation Period	Times: Proofing Period	Weights: Dough at Mix	Weights: Dough M'lded	Weights: Loaf Hot	Weights: Loaf 1 hr.	Weights: Loaf 18 hrs.	Volumes	Score
No.	%	min.	min.	grams	grams	grams	grams	grams	c c	
1009	59	255	60	564	518	457	448	435	2160	100
1001	58	231	60	543	539		488	460	2010	99
1008	60	203	53	542	535	500	492	464	2000	97
1002	58	225	53	532	527	491		462	1880	95
1012	59	225	54	537	527	489		460	1870	91
1006	61	195	55	541	536	494	486	471	1735	91
1005	59	187	65	536	531	496	484	464	1820	90
1010	58	165	60	531	527	491	482	468	1760	83
1011	56	229	51	529	524	489	479	459	1720	76
1003	53	186	57	520		473	464	438	1650	63
1013	58	224	51	528	532	496	487	468	1630	56
1007	65	165	46	557	552	512	500	478	1460	46
1004	58	192	45	549		504	498	473	1415	35
1014	59	206	43	540		497	490	466	1295	32

Clarification. The chief objection to the application of autodigestion for the measurement of diastatic power has been due to the colloidal character of the flour-water or malt-water suspensions. The longer the digestion continues the greater the degree of dispersion of these colloidal protein and dextrin products. Considerably difficulty has been encountered in obtaining a clear solution of sugars from enzymatic action and sufficiently free from those colloids which interfere with quantitative sugar determinations. Lead acetate as a clarifying agent for flour or malt solutions is exasperating in its slowness and poor results. It was found to be practically worthless for this work because of its failure to stop diastatic action.

The previous inhibition of enzymatic activity by acid or alkali is not satisfactory because of the necessity of again neutralizing the solution before adding the lead reagent.

The first obstacle to overcome was, therefore, that of clarification. The literature supplies numerous methods for investigation. Blish, (1918) made a study of protein precipitants and reported that "reagents ordinarily used for precipitating proteins, such as alcohol, acetic acid, trichlor-acetic acid, salts of heavy metals, colloidal iron, aluminum hydroxide cream, phosphotungstic acid, and tannic acid, are for various reasons unsatisfactory for removing gliadin from water extracts of flour." He recommended tenth normal copper sulfate and sodium hydroxide as the most efficient precipitant of protein nitrogen. Phosphotungstic acid appeared to be the most serviceable for rapid work, and was the reagent used by Swanson and Calvin (1913) and by Thatcher and Koch (1914), for the clarification of their flour suspensions and extracts. The excessive cost, however, prohibits its use in quantity for control and service laboratories when other reagents can be substituted. Folin and Wu (1919), developed a new protein precipitant, tunstic acid, which they applied to the precipitation of blood proteins. After the addition of one volume of ten percent sodium tungstate ($Na_2WO_4 \cdot 2H_2O$.), to diluted blood serum they added, with shaking, one volume of two-thirds normal sulfuric acid. The resulting precipitate was in such form that it could be easily centrifuged and filtered. The acid is intended to set free the whole of the tungstic acid and to neutralize the carbonates usually present in the commercial tungstate, with about ten percent in excess. Because of the efficiency and ease of application, combined with a greatly reduced expense, it would seem that this reagent deserves a wider application in the clarification of colloidal protein suspensions than it enjoys at present. A few trials on flour suspensions, extracts, and malt syrups gave promise of its being better suited to these than any other method yet employed.

Preliminary experiments showed that 2. cc of a 15% sodium tungstate solution were sufficient for the soluble proteins in 5 grams of flour, or 3. cc for 10 grams. These preliminary trials did not always result in a good clarification even though the equivalent amounts of 2/3 N. H_2SO_4, as suggested by Folin and Wu, were employed. Sometimes the supertant liquid became clear almost immediately, the flocculated proteins settling out rapidly, and again the cloudiness persisted after a half hour's centrifuging. The reason was not far to seek. The addition of the sodium tungstate to a suspension of sound flour in water produces an alkaline reaction. The complete precipitation of the proteins from a colloidal suspension depends upon their adsorption to the tungstate ion and the subsequent precipitation of the coagulated aggregate by throwing the hydrogen ion concentration over sufficiently far to the acid side of the isolectric point. This was accomplished by Folin and Wu through the addition of 2/3 N.H_2SO_4 in quantity which neutralized the combined alkalinity of the tungstate and blood serum with a slight excess; but in the case of flour extracts the higher buffer value * of the phosphate and other salts present requires a much larger excess of acid to produce the necessary hydrogen ion concentration. A number of clarifications were obtained in which the supernatant liquid became as clear as water after a few minutes centrifuging. The hydrogen ion concentration of these solutions was determined electrometrically and they were found to have values in terms of pH ranging from 2.117 to 1.337. ** The slightly cloudy liquids from the unsatisfactory clarifications all showed pH values of 2.67 or above.

These and subsequent results confirm the fact that the success of the sodium tungstate clarification depends upon proper acidification of the Na_2WO_4 protein suspension to a hydrogen ion concentration of $1. \times 10^{-2}$ or more, corresponding to a pH of 2.0 or less. There is no danger of precipitating the colloidal tungstic acid hydrate even with a much larger concentration of acid. Instead of the 2/3 H_2SO_4 of Folin and Wu, or the 1. N. acid which was first used in these experi-

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*(The term "buffer" was introduced by Fernbach and Hubert (Comptes rend. Acad. Sci. 131,293 (1900). It was repeatedly used by Sorenson (Comptes rend. du Lab. de Carls. 8,53 (1909), (Ergebnisse der Physiologie 12,-523 (1912), by Henderson (Ergebnisse der Physiologie 8,254 (1909) and by Jenny Hempel (Comptes rend. du Lab. de Carls. 13,1 (1917) to designate the effect of various salts in the media which dissociate upon the addition of acids or alkalies and therefore "use up" different amounts of the titrating reagent before significant changes are brought about in the hydrogen ion concentration of the media. The importance of these buffer salts in wheat flours has been emphasized by Bailey and Peterson (Jour. Ind. Eng. Chem. 13,916 (1921).

**(All electrometric determinations were made with the Leeds and Northrup Type K Potentiometer, using the Bailey (1920) hydrogen electrode. The pH values corresponding to the millivolt readings were taken from the Schmidt and Hoagland tables.)

ments, concentrated sulphuric acid added from a micro-pipette appears to be more efficient in precipitating the protein-tungstate. The precaution of adding the acid slowly, drop by drop, with shaking of the solution must be observed, otherwise a local concentration will produce a precipitation of flocks of colloidal tungstic acid, and with further danger of decomposing some of the carbohydrates. Thymol Blue (Clark 1920) with its acid range at pH 2.+ serves as a very convenient indicator for the first few trials with any new sample, since it is necessary to add only two or three drops of the concentrated acid in excess of the pink color to produce the proper acidity for complete precipitation. After a few trials uniform results were always obtained by measuring the acid from a 1. cc micropipette or counting the number of drops required to produce the proper color by Thymol Blue.

Because of their higher buffer value malt flour suspensions are found to require a slightly larger amount of acid. In this connection a difficulty was later encountered in the clarification of suspensions to which considerable quantities of acid had already been added. The same principle of colloidal protein precipitation applies here as well, and the clarification is satisfactory if the suspension is first neutralized by means of a few drops of strong NaOH. The Thymol Blue, alkaline range, blue color (pH 8.0 to 9.6) likewise serves for this point, and though the hydroxyl ion concentration does not need to be carried so far, it does no harm as the subsequent addition of acid brings it back immediately to a pH of 2.0 or less.

The suspensions to be clarified in this series were always centrifuged for two or three minutes to save time, and more especially to form a compact mass of flour solids in the bottom of the centrifuge tube from which the clear supernatant liquid could be poured or pipetted without stirring up any of the material which had been thrown down. But if a centrifuge is not available the starch and precipitated proteins settle out clear in about five minutes, and if desired the supernatant liquid can be rapidly filtered through a fine quantitative filter paper. This is especially true of the clarified dilute solutions of malt extracts intended for sugar determinations. As will be shown later, however, the sodium tungstate clarification followed by a few minutes centrifuging effects the elimination of all filtrations, which heretofore have required hours, and which because of the errors thus introduced have been the stumbling block in many investigations of this nature.

The amount of soluble nitrogen remaining in solution was determined by the Kjeldahl method before and after clarification, using different amounts of tungstate and varying acidities. The residual nitrogen appears to reach a fairly constant minimum for the flour used

under the conditions of clarification described above. Different materials show different content of solublie amino nitrogen and ammonia nitrogen which is not removed by the tungstate procedure, but which shows no vitiating effect on the reducing sugar determinations. The results of the above discussion are summarized in table VII, which shows the nitrogen remaining in solution after treatment with sodium tungstate.

TABLE VII.

The efficiency of the Sodium Tungstate reagent as a clarifying agent for flour suspensions at various concentrations and acidities.

Flour grams	Final Volume of Clarified Solution cc.	Na_2WO_4 15% cc.	Acid Added	pH of resulting Solution	Nitrogen Remaining in 100 cc's of Solution grams	Remarks on Clarification
5	100	4	5. cc ⅔N H_2SO_4	3.88	.0034	Fair
5	100	2	2.5 cc N/1 H_2SO_4 10 drops in excess by Thymol Blue*	2.603	.0021	Excellent
5	100	3	Acid by Methyl Orange	5.04	.0129	Poor; very cloudy
5	100	2	10 drops in excess by M. O.	3.446	.0027	Good
5	100	2	Acid by Thymol Blue*	2.536	.0021	Very good
10	200	3	4. cc N/1 H_2SO_4	2.117	.0026	Excellent
10	200	3	.4 cc Conc. H_2SO_4	1.468	.0021	Excellent after 1 hr. digestion
10	200	3	.4 cc Conc. H_2SO_4	1.457	.0021	Excellent after 3 hrs. digestion
10	200	0	0. cc	5.778	.0241	No clarification after 1 hour digestion.
10	200	0	0. cc	5.596	.0379	No clarification after 3 hours digestion

*(7. drops Thymol Blue Indicator).

Reducing Sugars. Several methods present themselves for the determination of reducing sugars in the clarified solutions from flour, or malt enzyme digestions, and different ones have found favor with the different workers on the products of diastatic action. Swanson and Calvin (1913) Thatcher and Koch (1914), and Thatcher and Kennedy (1917), applied the iodine titration method to the residual copper, after the Fehlings reduction, basing their work on the articles published by A. W. Peters (1912). Spoehr (1919) modified the details of the same method for application to small amounts of sugar-containing juices from cacti. He found that his solutions contained other substances than sugar which contaminated the weight of reduced cuprous oxide and so made it necessary to determine the unreduced copper. Harter (1921) followed the Clark (1918) modification of the Scales (1915) procedure to measure the reducing sugars produced by the diastâse of Rhizopus tritici. The method of Bertrand (1910), which determines the amount of copper reduced by the sugars, has been but little used in this sort of work. The Bertrand titration method furnishes a convenient way of checking the weights of the precipitated cuprous oxide, and was so used in this laboratory to determine the accuracy of the crucible weights as compared to the actual copper reduced. In this way it was found that the supernatant liquids from the tungstate clarification as carried out in the present investigation gave accurate and concordant results by weighing the filtered cuprous oxide precipitate, and the difficulty in contamination of the reduced copper by non-sugars, as recorded by Spoehr, was therefore not encountered.

Because of the conditions under which this work was done, it was found more convenient to use the prepared gooch crucibles with asbestos mats for filtering the reduced copper than to apply the iodine method of titration, with its consequent necessity for the preparation of solutions. The total time required for the preparation and weighing of the gooch crucibles is hardly greater than that for the titration procedure. Furthermore, the excellent contribution of Shaffer and Hartman (1921) has shown that the iodometric titration method for residual copper as previously carried out is not without its possibilities of serious error. On the other hand Quisumbing and Thomas (1921) in their recently published article on the reduction of Fehling solution by different sugars, point out several possible sources of error in the reducing sugar determinations according to the official Munson-Walker procedure. Unfortunately these two articles did not appear until the work reported here was well along toward completion, and it was believed best to complete the determinations using the same comparative procedure; consequently all reducing sugar determinations reported in the experimental part of this paper were made by

the Munson-Walker method, (A. O. A. C. Methods of Analysis, revised, 1919. Section VII.) A battery of forty No. 0 porcelain gooch crucibles were used, and the cuprous oxide resulting from the Fehlings reduction was filtered onto thick asbestos mats, washed, dried and weighed. The corresponding weights of maltose were taken from the Munson-Walker tables. The crucibles were fitted with mats of properly prepared washed and ignited asbestos, at least 1 cm. in thickness, and washed into place by whirling with a stream of hot water from a wash bottle. They were then washed with 95% alcohol and dried at 100° for 1 hour. The weights of the crucibles so prepared remained constant in the dessicator for periods of weeks.

The Fehling solutions used showed no auto-reduction in the blanks. The only standardization necessary was to run a few blank determinations in order to apply a correction for each new lot of asbestos prepared. The 400 cc Pyrex beakers used for Fehling solution reductions were of approximately uniform thickness, the watch glasses used for covers fitting rather closely. The flame was so adjusted that the different samples started boiling usually within five seconds of the four minute period stipulated. The chief source of error was found to be in the loss of the weight shown by different lots of prepared asbestos upon pouring the hot Fehling solution through the mats. The maximum variation seldom went beyond 1 milligram and with triplicate determinations the weights of Cuprous oxide usually checked within .2 to .3 milligrams of the average. It would appear highly desirable for this sort of work to combine the method of Shaffer and Hartmann, and of Quisumbing and Thomas into a standard method in which the sources of uncontrolled error are reduced to a minimum. Also the application of sodium tungstate as a clarifying agent for protein-containing solutions could be profitably studied in its connection with such a method.

Effect of Clarifying Agent on Sugar Determinations. The next point requiring investigation was the effect, if any, of various amounts of the sodium tungstate, as used for clarification, on the determination of reducing sugars by Fehling solution. Preliminary trials on flour-water suspensions with and without added dextrose indicated that the addition of the sodium tungstate in excess for clarification did not affect the Fehling's reduction. To confirm this point and to see what results could be obtained from unclarified solutions, the following method was used: five grams of a flour showing considerable diastatic activity were weighed into 250 cc beakers, 50 cc of water at 27°C were added, and the mixture thoroughly stirred. In some cases 40 cc of H_2O and 10 cc of a 0.5% dextrose solution were added in place of the water alone. These were allowed to digest for one hour at 27°C, stir-

ring at intervals. Some of the samples were then clarified by Na_2WO_4, using Thymol Blue as indicator. In the other samples the enzymic activity was inhibited by 5. cc of 0.2N NaOH. After clarification, or inhibition of the enzymic action, the solution was diluted to 100 cc in a volumetric flask, poured into a 100 cc centrifuge tube and whirled ten minutes. 50 cc of the clear supernatant liquid were thern pipetted out into a 400 cc beaker for determination of sugars by the Munson-Walker method.

The results are compiled in table VIII under three groupings, samples numbered 1 to 4 include the blanks, 5 to 7 the flour with no added dextrose, and numbers 8 to 13 the flour samples with a known weight of dextrose added.

TABLE VIII.

The determination of reducing sugars in solutions with and without clarification by sodium tungstate.

Sample Number	Flour grams	Dextrose Added milligrams	15% Na_2WO_4 For Clarification cc.	Cu_2O Weighed grams	Average Weight of Cu_2O grams	Expressed as Dextrose Recovered milligrams
1.	0.	0.	0.	-.0005 -.0006	-.0005	none
2.	0.	50.	0.	.1146 .1150	.1148	49.7
3.	0.	0.	2.	-.0004 -.0002 -.0006	-.0004	none
4.	0.	50.	2.	.1138 .1156 .1140 .1145	.1145	49.6
5.	5.	0.	0.	.0800 .0788 .0795	.0794	34.0
6.	5.	0.	2.	.0795 .0782 .0795 .0800	.0793	34.0
7.	5.	0.	2.	.01665*	.01665	6.8
8.	5.	50.	0.	.2008 .2004	.2006	89.0
9.	5.	50.	1.	.1985 .1987	.1986	88.6
10.	5.	50.	2.	.1997 .2004 .1963 .1963	.1982	88.4
11.	5.	50.	5.	.2013	.2013	89.4
12.	5.	50.	10.	.2027	.2027	90.4
13.	5.	50.	2.	.1975 .1984	.1980**	88.0

*Average of 5 determinations.
** (Filtered)

The values recorded as sample number 7 are the average found for five determinations of the natural reducing value of the flour, the diastase having been inhibited by preliminary tungstate and acid treatment.

The determinations recorded in Table VIII were obtained under conditions of poor temperature control. The De Khotinsky electrically heated constant temperature water bath with electrostatic control which was used for all subsequent work had not yet been installed, and these digestions were made at 27°C in an incubator. The temperature fluctuated rather widely (around .5 degree), and the consequent variation in diastatic activity was to have been expected. This series was not repeated as such, because later data obtained in connection with other experiments has shown that the conclusions as indicated by Table VIII are valid, namely; (1) The use of 15% sodium tungstate in quantities up to 5 cc, and sulfuric acid for clarification of flour suspensions, neither interferes with, nor affects the determination of reducing sugars in the clarified solution by the Munson-Walker method. (2) It is not absolutely necessary to clarify the supernatant liquid obtained from the centrifuging of a 5% flour and water suspension. This should be further qualified by notes on the appearance of the Fehling's reduction. It was shown in every case that clarification was an advantage in the reduction of Fehling's solution, especially in the case of cloudy solutions. If unclarified, the boiling Fehling solution foamed badly, and the formation of Cu_2O appeared to be hastened by the coagulated protein, resulting in a dark and somewhat muddy looking precipitate which filtered badly, and showed a tendency to adhere to the surface of the beaker. (3) The use of the sodium tungstate clarifying reagent renders the solution **clear and protein-free, and when centrifuged to throw down suspended matter it eliminates all necessity for filtration.** Further proof that filtration of the clarified centrifugate is entirely unnecessary, is evident from data obtained in other experiments, at different times and for different purposes, but using the same flour sample and method. The object in view when these samples were run bore no intentional relation to conclusion (3), but the results substantiate those obtained in table VIII. This data is collected and tabulated in Table IX.

TABLE IX.

The determination of reducing sugars in the centrifugate from the sodium tungstate clarification before and after filtering.

Sample	Grams Cu_2O	
1 Filtered	.1259	
2 Filtered	.1259	.1256 Average
3 Filtered	.1252	
4 Unfiltered	.1260	
5 Unfiltered	.1251	.1255 Average
6 Unfiltered	.1256	

On the other hand, a very few trials are sufficient to convince one that filtration of the unclarified solution from a flour-water suspension is a most unsatisfactory procedure and should be expected to give inaccurate results for reducing sugars.

A further careful scrutiny of the data in table VIII brought out a peculiarity in the determinations which has not yet been satisfactorily explained. Comparing samples Nos. 5, 6 and 7, and Nos. 1, 4 and 7, it appears that there is an increase of reducing sugars as determined by weighing the Cu_2O over and above that added in the form of dextrose. This question, like several others which were brought out in the course of this series of investigations, is recorded here with whatever explanation appears possible from the data at hand, with the hope of enlisting the interest and experimental efforts of other workers. The most obvious explanation is that the natural reducing value of the flour extract is due (probably) to dextrose, while the increase of reducing power after one hour of diastatic activity is due to maltose. In table VIII the weights of Cu_2O as recorded are produced in some instances by dextrose alone (samples numbered 1 to 7 inclusive), and in others by maltose alone or by both dextrose and maltose (samples 8 to 13 inclusive).

TABLE X.

The relation of actual to calculated values for the reducing power of dextrose added to autolytic digestion of diastatic flour.

(Data taken from Table VII).

	A In terms of Cuprous Oxide Milligrams	B In terms of Dextrose and Maltose Milligrams
Without added dextrose		
Total reduction after 1 hour's diastasis (samples Nos. 5 and 6)	79.35	60.88 = Maltose
Blank (natural reducing power of flour (sample No. 7)	16.65	6.86 = Dextrose
Difference due to diastasis	62.70	54.02 = Maltose
With added Dextrose		
Dextrose added (sample Nos. 2 and 4)	114.58	49.83 = Dextrose
Blank (natural reducing power of flour (sample No. 7)	16.65	6.86 = Dextrose
Total, due to Dextrose	131.23	56.69 = Dextrose
Total reduction after 1. hour diastasis (samples Nos. 8 to 10 inclusively)	199.51	
Total reduction due to Dextrose	131.23	56.69 = Dextrose
Difference due to diastasis	68.28	52.12 = Maltose
		108.81 = Total reducing sugar
Value found	62.70	108.81
Value calculated	68.28	110.77
Increase	5.58 mg.	2.96 mg.

In Table X the data has been rearranged to show that the increased weight of cuprous oxide, in the range of values between 63 and 131 milligrams, is equivalent to a positive error of 5.58 milligrams of dextrose, or an increase of nearly 9% over the calculated value. Since the average error of the method, due to slight differences in temperature, volumes and weights is about 0.8 milligram of Cu_2O, the above variation is more likely due to other causes. When the data is recalculated into terms of dextrose and maltose respectively, as shown under Part B of Table X, the error is reduced somewhat, due to the smaller numerical value of the corresponding reducing sugars. This method of calculation gives a difference of approximately 3. milligrams of reducing sugars, or about 5. per cent. Differences of similar magnitude, and positive in sign, have been obtained in subsequent work.

Adsorption by Starch. The next explanation to suggest itself is a displacement concentration due to the volume occupied by the 5. grams of flour in the 100 cc flask. It will be shown that this accounts for only part of the error. The hydrated colloids of the flour, e. g. starch and gluten, might be expected to adsorb some of the sugars, thus reducing the concentration of the supernatant liquid. Thus there would exist a ratio between the reduction in the concentration of sugars due to adsorption, and the increase of concentration due to volume displaced by the flour. The difference then in result between the dextrose recovered, and the dextrose added to the flour suspension plus the reducing value of flour-water suspension, would be resultant of this ratio of $\frac{\text{Displacement}}{\text{Absorption}}$ x concentration.

We should also expect these colloids to be sensitive to changes of electrolytes or of hydrogen ion concentration, yet the results of several trials did not show sufficient adsorption of dextrose by flour colloids in excess water at a pH of 6 or less to be recognized by the methods employed. On the other hand, the errors were always in the opposite direction, that is, there was always an increase in sugars recovered over that expected.

Samples of both wheat flour and wheat starch were weighed into accurately graduated flasks, the flasks fiilled to the mark with distilled water, and allowed to stand either one hour or 24 hours, before being brought again to volume and weighed. From these weights it was calculated that .5601 cc of water is displaced per gram of flour, and .7086 cc per gram of starch. Using these values to calculate the displacement concentration, the total increase in weight of the Cu_2O, due to increased concentration, should be approximately 2.2 milligrams. This is less than half the difference actually obtained. For

want of a better explanation the remaining error might be laid to the increased reduction of the Fehling solution by the addition of dextrose to the maltose in solution. Such a probability is suggested by the recently published work of Quisumbing and Thomas (1921).

Preparation of Wheat Starch. This increased recovery of reducing sugars by Fehling's reduction is likewise shown by some of the data obtained on starch. To obtain some idea of the behavior of starch in flour under the conditions of these experiments, several samples of wheat starch were washed out of flour with running water, the gluten being held back by manipulating over a No. 10 flour silk gauze. Repeated washing, decantation, and differential centrifuging failed to remove all the protein matter from the starch, which still showed some slight diastatic activity after driying. Other samples were then prepared and the proteins removed by dispersion and washing with very dilute NaOH. Too strong soda solution gelatinizes the starch, rupturing the granules and even though a concentrated alcohol treatment retrogrades it back to insoluble starch (Herzfeld and Klinger, 1921.), the physical and chemical character of the material has been changed, and it is no longer comparable to the raw wheat starch of the flour. After the proteins and NaOH have been washed out with distilled water, the starch is carefully centrifuged, and only the center portion of the column deposited in the bottom of the centrifuge tube is taken. This is repeatedly washed with cold distilled water containing a few drops of HCl per liter until no more acid is removed from the solution. The material is then filtered on a Buchner funnel, washed with re-distilled neutral alcohol, and dried in a vacuum at 105° for five hours. After cooling in a dessicator, the samples were allowed to reabsorb moisture from the atmosphere for several days, and bottled. This prepared starch could then be weighed out in the open balance without errors due to rapid changes in weight by moisture absorption. Its moisture content was determined. These starches showed no diastatic activity nor reducing sugars.

Samples of this air dried starch were weighed into 250 cc beakers, and 50 cc of water added to each at 27° centrigrade. Ten cubic centimeters of 1% dextrose were then pipetted in, well stirred, and allowed to remain at 27°. At the end of exactly one hour they were diluted to 100 cc in a calibrated volumetric flask, centrifuged clear, and 50 cc of this clear supernatant liquid taken for determination of reducing sugars. To determine whether the change of acidity by the Na_2WO_4 clarification procedure would affect the adsorption of sugars by starch, and to check up on the effect of clarification upon Fehling's solution reduction (table VIII, page 33), alternate samples were clarified at the beginning of the digestions. In other words 3. cc of Na_2WO_4 (15%)

and .4 cc conc. H_2SO_4 were added immediately after the 10 cc of dextrose solution. The results are shown in table XI.

TABLE XI.

The recovery of reducing sugars after addition to starch in water.

Number	Starch as Substrate Grams	pH	Sugars Added Milligrams	Sugars Recovered By Analysis Milligrams	Sugars Recovered After Correction For Volume Displacement Milligrams
			Dextrose		
1.	3.0	5.092	48.4	50.4	49.2
2.	3.0	1.790	48.4	49.9	48.8
3.	4.0	5.000	24.2	25.2	24.5
4.	4.0	1.618	24.2	24.7	24.0
5.	5.0	5.581	48.4	50.4	48.5
6.	5.0	1.932	48.4	50.5	48.7
7.	7.0	5.466	48.4	53.5	50.7
8.	7.0	1.900	48.4	51.5	48.6
			Maltose		
9.	4.0	4.600	40.9	43.8	42.4
10.	4.0	1.615	40.9	42.8	41.5
11.	4.0	1.900	61.4	64.9	63.0

The last column of figures in table XI shows the calculated weights of Cu_2O after correcting for the concentration displacement by the starch in 100 cc volume. The corrected values fall almost within the limit of experimental error with the exception of samples numbers 1, 7, 9 and 11. The good agreement of the pairs of results also confirms a conclusion of table VIII, namely, that the Na_2WO_4 clarification has no effect on Fehling solution reduction. It is also evident from the data given in Table XI that there is no adsorption of reducing sugars by the starch.

Inhibition of Enzyme Activity. Much has been published on the effect of the so-called catalytic poisons on various enzymes. The results are so numerous and the conclusions so contradictory that it would serve no purpose to review them at length. It has been shown that each enzyme poison, whether it be an antiseptic, or heavy metal salt, must be conisdered separately and in its relation to the particular enzyme in question. In general, the salts of the heavy metals, antiseptics, and in fact most of the so-called catalytic poisons, have but little inhibiting effect on the activity of diastase under normal conditions of pH and salt concentration except as they are able to precipitate the enzymic carrying protein or to influence the pH of the medium. The work of Sherman and Caldwell (1921), has further emphasized the protective action of the amino acids on the enzymes in the presence of catalytic poisons. Many of those who have investigated diastatic activity have recorded the use of NaOH in varying concentrations to stop enzymic action. Others have employed low temperatures. The

effect of continually increasing amounts of alkali on diastatic digestions is shown later in Table XVI and Fig. 3. The concentration of alkali required for complete inhibition of flour diastase is considerably higher than that generally recomended, and there would seem to be serious danger of destroying some of the sugars present by the addition of such hydroxyl ion concentration, as pointed out by Neff, (Armstrong, 1919.). Furthermore, the increased dispersion and solution of proteins by the added NaOH is not desirable. The unsatisfactory results obtained in preliminary experiments, whenever NaOH was used to stop diastatic activity, or lead acetate for clarification of flour suspensions, in contradistinction to the uniformly satisfactory results by the use of sodium tungstate and sulphuric acid, lead to the experiments which are summarized in Table XII.

Ten grams of flour were weighed out into 300 cc flasks and 100 cc of distilled water added by pipette, with continual shaking to get the flour thoroughly stirred throughout the liquid. The various materials to be used as inhibiting agents were then added and the whole allowed to remain in the water bath for the lengths of time as designated in the table, (shaking up at fifteen minute intervals). Sample number 1 was clarified by the tungstate reagent at the end of the first hour. Sample number 2 was clarified at once, then allowed to stand one hour before determining the reducing sugars. Sample number 3 was clarified in the same manner as number two, except that it was allowed to stand two hours before determining the reducing sugars. Sample four was tried with only 3. cc of sodium tungstate, omitting the precipitation by sulfuric acid until the end of the hour. Samples five and six show the effect of two different concentrations of alkali previously recommended in the literature for the inhibition of diastatic activity. Samples seven and eight were treated with 2. cc of basic, and 2. cc of neutral lead acetate, respectively, and allowed to stand one hour before filtration and removal of the lead for sugar determinations. They were then clarified and treated according to the official A. O. A. C. procedure. Sample number 7 was cloudy and required twelve hours for filtration, while sample eight filtered much more rapidly. Samples nine and ten were cooled to zero degrees by an ice and salt bath. In samples 1, 5, 6, 9, and 10, the diastatic activities were stopped by the sodium tungstate clarification in the manner described above, while sample 4 required the addition of .4 cc concentrated H_2SO_4. After clarification the samples were all made up to a volume of 200 cc, centrifuged, and 50 cc aliquots taken for reducing sugar determinations. The results are expressed in table XII in terms of cuprous oxide corresponding to the reducing sugars from 50. cc of the clarified solution (2.5 grams flour.)

TABLE XII.

The effect of different inhibiting agents on the diastase of flour.

Sample No.	Inhibiting Agent	Time and Temperature	Weight Cu_2O per 50. cc's of Solution (2.5 g. flour)	pH of Digestion
1.	none	1 hr. at 27°	.0833	......
2.	(Na_2WO_4 +H_2SO_4)	1 hr. at 27°	.0140	below 2.0
3.	(Na_2WO_4 +H_2SO_4)	2 hrs. at 27°	.0141	below 2.0
4.	(Na_2WO_4 only)	1 hr. at 27°	.0491	7.241
5.	2. cc 0.1N. NaOH)	1 hr. at 27°	.0316	......
6.	(5cc 0.1N. (NaOH)	1 hr. at 27°	.0169	9.1336
7.	PbAc (Neut.)	1 hr. at 27°	.1034	......
8.	PbAc (Basic)	1 hr. at 27°	.0066	......
9.	Ice	1 hr. at 0°	.0287	5.981 (21.°C)
10.	Ice	3 hrs. at 0°	.0388	5.981 (21.°C)

Confirmatory results similar to these and leading to the same conclusions have been obtained on two other flours (Our No. 1 and 1003).

Samples numbers 2 and 3 of Table XII, as examples typical of many similar determinations, show conclusively that the use of sodium tungstate and sulfuric acid, with a final pH of between 1.5 and 2, effectually stop diastatic activity. This is accomplished both by complete precipitation of the enzymic active protein and by effecting a hydrogen-ion concentration which itself inhibits diastatic activity. The concentration of acid is not sufficient to further hydrolyse the disacharides or dextrins, even after several hours standing. This had been previously pointed out by Swanson and Calvin, who used a final concentration of .02N H_2SO_4, which corresponds to a pH of somewhere between 2 and 2.5. This fact allows an accurate measurement of the reducing sugars by gravimetric Fehling's determination at the operator's convenience.

Samples 5 and 6, Table XII, indicate that NaOH is not a reliable inhibiting agent to use for diastatic preparations where the quantity of buffer is as high as that found in flours or malts, unless a large concentration is used. There is also the probable danger of destroying

part of the reducing sugars present if too high a concentration of alkali be used.

The use of lead acetate, (table XII, numbers 7 and 8), gives variable results depending upon the concentration of the reagent, the concentration of the material used to de-lead the solution, and the time consumed in the operations of filtration.

In point of time alone the sodium tungstate procedure requires only about five minutes, with the elimination of all filtration, which is a great advantage in enzyme work, while the clarified solution can be allowed to stand for several hours, or until all of the samples are ready for the Fehling reduction.

Samples numbers 9 and 10 (table XII), clearly indicate that the temperature of an ice bath will not stop diastatic activity in solutions where the diastase is protected by buffers naturally occurring in the plant medium. In the two trials the temperature within the flasks was held at zero by means of salt and ice, and they had to be stirred at short intervals to prevent ice crystals forming on the inside surface of the flasks. Other samples with temperatures around 5°C showed very nearly the same results. Samples 2 and 3 (Table XII), in conjunction with results of nearly a hundred other similar determinations, serve to show this "blank" gives very constant and duplicable results, and affords a measure of the reducing sugars present in the flour. While this reduction of Fehling solution is due to dextrose, and the total reduction by autolytic digestion of the flour-water suspension is a result of added maltose produced by diastatic action, the difference in the weight of Cu_2O produced give a reliable measure of the maltose. This procedure for the determination of original reducing sugars in the sample should be directly comparable to the values obtained from the official alcohol extraction method. Likewise, it should be possible to substitute the sodium tungstate clarification for the lead acetate procedure in the alcohol-extract of sugars from flour and malt products. Preliminary experiments on the application of this new procedure for both reducing and total sugars have indicated its successful application. Collaborative work with the Department of Agriculture, Bureau of Chemistry, is now being conducted on this problem and the results will be reported at a later date.

Effect of Concentration. In general, investigators of enzymic activities have found it necessary to take the concentration effect into consideration before establishing their conditions for observations and measurements on enzyme preparations. This concentration effect is especially important when working with extracted and purified enzyme preparations. In the preliminary discussion of factors which may limit the activity of diastase in one way or another two such fac-

tors were considered, namely, amyloclastic power, and variable resistance of different starches to amyloclastic action.

As a third limiting factor the concentration effect on diastatic action should receive attention because of its peculiar relation in this series of measurements. Before diastatic enzymes can act to best advantage there must be sufficient water present. In the germinating seed this is assured and controlled through imbibition of water by the colloidal system. In a dough batch the concentration is likewise controlled by the amount of water the flour colloids can imbibe and still retain sufficient elasticity and tenacity to handle well in baking practice. (Ostwald 1919, and Luers and Ostwald 1919-1921). This degree of concentration varies considerably with different flours and in the hands of different bakers, within certain limits. When compared to the long period of time required for germination, the short fermentation of a dough should show concentrations which are more or less comparable.

To determine what effect concentration would have on the activity of diastase as measured by the methods employed in this report, two types of experiments were carried out. In one, attempts were made to measure the reducing sugars produced in a dough fermentation containing percentages of water comparable to the normal absorption as obtaining in bake shop practice, and in the other, to determine the concentration effect in dilutions such as are used in the method finally chosen for measurements of comparative diastatic powers in the standard flour samples.

In Table XIII the first three values given are those for diastatic activity in the doughs, and were obtained as follows: 50 grams of flour were weighed into 250 cc beakers and allowed to come to a temperature of 27°C. Distilled water at 27°C was added from a burette in volumes ranging from 27.5 cc to 50 cc, or in other words in amounts corresponding to absorptions of 55 to 100%. The flour and water was thoroughly doughed up by means of a stiff spatula and the stiffer doughs were kneaded a minute in the fingers. This dough was pressed into the bottom of the beaker, covered, and placed in the water bath of 27° for one hour. At the end of that time the dough was weighed and one-fifth of the total weight, corresponding to ten grams of flour, was taken for determination of sugars. This aliquot sample of dough was transferred to a 250 cc bottle, 100 cc of water were added, and several drops of 50% NaOH to aid in dispersing the gluten and slow up enzymatic activity. The bottle was quickly stoppered and shaken for a few minutes to completely disperse the gluten and dissolve out the sugars. The contents of the bottle were then rinsed into a 200 cc volumetric flask and clarified by sodium tungstate

and sulfuric acid as described above. From the centrifuged supernatant solution 50 cc aliquots were pipetted out for reducing sugar determinations. The values for the other four concentrations as recorded in the same table were obtained by varying the ratios of the flour-water suspensions from 1 : 2.5 up to 1 : 40. and allowing the mixture to digest autolytically for one hour at 27°. The diastase in these samples was then stopped by the regular sodium tungstate procedure, made up to volume, and the reducing sugars determined in the centrifugate in the same manner as for the other three samples.

TABLE XIII.

The effect of varying ratios of flour to water on the diastatic activity of a wheat flour.

Sample Number	Weight of Flour Grams	H_2O Volumes cc	Ratio	Weighed as Cu_2O per 2.5 Grams Flour 10. Grams	Calculated Weight of Cu_2O per Gram Flour Grams
1.	50.	27.5	1:0.55	.0675 .0665	.2680
2.	50.	35.0	1:0.70	.0727 .0723	.2900
3.	50.	50.0	1:1.0	.0862 .0853	.3430
4.	10.	25.0	1:2.5	.0835 .0835 .0827	.3348
5.	10.	75.0	1:7.5	.0838 .0831 .0836	.3340
6.	10.	100.0	1:10.0	.0833 .0836 .0830 .0839	.3338
7.	10.	400.0	1:40.0	.0835 .0835 .0820	.3340

The low results for samples numbers 1 and 2, table XIII, are probably due to an insufficiency of water to allow complete enzymatic activity. The normal dough for this flour would require 29.5 cc of water for 50 grams of flour, which is less than the amount added to sample number two. It must be understood, therefore, that diastatic action is **not complete** in a normal dough under average bake shop conditions. The result for diastatic activity in sample number three on the other hand, is too high by about 3. percent, yet it falls within the range of values as determined by the other samples. Considering, however,

the necessary inaccuracies of method in controlling temperatures, in doughing up the flour samples, in weighing and dividing the dough, and especially in the time required to disperse the dough and dissolve out the sugars before the enzymic activity can be inhibited by clarification, the agreement with the samples numbers 4 to 7 is about as close as could be expected. Attention might be called to the fact that the results as expressed in column five contain the errors of the actual determinations multiplied four times.

The excellent agreement of the results for autolytic digestion of the flour with water in ratios of 1:1 or more, over so wide a range of concentrations, eliminates one of the most troublesome factors in a determination of this nature. It enables one to add other reagents or to vary the total valume of the solution at will and yet have only the effect of the one desired variable imposed on the results.

It is evident here that the enzyme and its substrate exhibit an intimate relationship. The large and practically constant excess of starch substrate is available for the relatively small amount of enzyme which exists within the plant material constituting the flour particles and which is held in close contact with the starch granules on which it must act. When sufficient water has been added to properly hydrate the bio-colloids and enable the enzymes to function, any further increase in the amount of water has but little effect, the enzymes having been adsorbed to the substrate and not immediately subject to dilution. This is not the case with the solutions of "purified" and dissolved diastatic preparations or extracts, which do show some concentration effect because of their suspension throughout the solution. The results of Thatcher and Koch described above, as well as the work of nearly all of the other investigators of diastase, have shown that a part, never all, of the diastatic activity could be extracted from flour as a solution. But these extracts do not show normal, i. e. natural, diastasis as in the plant tissue. Frequent shaking and prolonged digestion are necessary for such a removal of enzymes. It should be stated here in this connection that too frequent and violent shaking of the flour-water suspensions used in this work decreased considerably the quantities of maltose produced per unit of time, and the supernatant liquid evidenced increasing diastatic power. It was for that reason that the suspensions were agitated only at fifteen minute intervals by rotating the containing flasks.

But by far the more important contribution of this set of results to the problem in hand rests on the demonstration that autolysis of flour-water suspensions in ratios around 1:10 as carried out in this series of experiments can be considered as furnishing a logical laboratory basis for the measurement of diastatic capacity of the flour when mixed in a

dough. The temperature is taken as the average accepted fermenting temperature for ordinary bake shop practice. The time may be controlled at will. The pH of the digestion mixture is controlled by the flour, usually between 5.7 and 6.1, and is close to that at which the dough fermentation normally begins. The acidity which develops as fermentation proceeds in the dough gradually increases to a pH value between 4.8 and 5.4, at which point the diastatic activity is at or very near its maximum. Sherman and Thomas (1915), and Sherman, Thomas, and Baldwin (1919), showed that the protective action and stabilizing effect of buffer salts on diastase is very marked both with respect to concentration and temperature. This buffer effect undoubtedly accounts in part for the stability of the enzyme rate by the method of measurement employed here. This is also shown further on by the data on relation of pH to diastatic activity.

The relations between autolytic measurements and diastatsis in the dough during a normal fermentation are discussed further in connection with measurements of maltose production in doughs.

It would seem advantageous then from the standpoint of sugar production to mix doughs with the maximum amount of water which they will carry and still work well. The machinery designed for the modern large baking plants will handle doughs with absorption as high as 70%, which allows practically maximum maltose production by diastatic action.

Method for Measuring Diastatic Power of Flour. As a result of the data obtained up to this point a definite method was developed for the determination of diastatic power in flour. This procedure was used with variations in time, temperature, or pH, as the case might require, for all the experiments which follow. 10. gram samples of flour are weighed out and transferred to 250 or 300 cc Erlenmeyer flasks. These are placed in a water thermostat and brought to a temperature of exactly 27°C. A flask containing a sufficient volume of distilled water is also placed in the bath and kept at 27°C for subsequent use. By means of a pipette 100. cc of the distilled water are run into the sample, while rapidly rotating the flask to obtain a thorough suspension of the flour. The last few cc's in the pipette are allowed to rinse the material down from the sides of the flask. The flask is quickly replaced in the thermostat, stoppered loosely, and allowed to remain exactly 60 minutes. A few minutes after starting the digestion the flask is rotated to stir up the suspension and hasten the equalization of temperatures, and the shaking is repeated at fifteen minute intervals. At the end of the digestion period the contents of the flask are quickly rinsed into a 200 cc volumetric flask, diluted to about 175. cc and clarified.

To clarify, first make sure that the solution is neutral or slightly alkaline. Five drops of 0.04% Thymol Blue serves as a convenient indicator, appearing cream-yellow in color when neutral, or blue when slightly alkaline. Add 3 cc of a 15% solution of sodium tungstate ($Na_2WO_4.2H_2O$)and mix thoroughly with the flour suspension. Then add, drop by drop, from a 1 milliliter graduated pipette, with constant shaking, sufficient concentrated H_2SO_4 to turn the indicator a decided pink color, with two or three drops in excess. Four-tenths of a milliliter (0.4 cc) are usually sufficient if the original flour suspension was nearly neutral. This clarification likewise serves to stop the enzymatic activity and prevents further change in sugar content. Dilute to the mark, shake thoroughly, pour into centrifuge cups, and whirl for about five minutes. By means of a calibrated pipette transfer 50. cc of the clear supernatant liquid to a 400. cc pyrex beaker for the determination of reducing sugars by the Munson-Walker official method.

A blank is also run at the same time as the sample to correct for the natural reducing sugars in the flour. It likewise gives a measure of these reducing sugars. To prepare the blank, mix 100. cc of water at 27°C and 10. grams of flour and immediately inhibit diastatic activity by clarifying with the sodium tungstate in the manner just described. The blank determination is then carried out in the same manner as the samples except that the addition of 0.4 cc of concentrated H_2SO_4 is omitted on dilution to volume. It does no harm to allow the clarified solution in the beakers to stand an hour or two until other samples are ready. When making the Fehling reduction the excess acidity of the solution can conveniently be neutralized by using a predetermined number of drops of strong NaOH. Following the reduction of the Fehling solution the Cu_2O is filtered, washed, dried, and weighed in the gooch crucibles. Subtract the weight of the Cu_2O corresponding to the blank from that of the sample. The result is the Cu_2O corresponding to the maltose produced by the diastase in 2.5 grams of flour. This value for anhydrous maltose as found from the tables, multiplied by 4. gives the diastatic power per 10. grams of flour. This procedure has been found by many repeated trials on different flours to give accurate results which could be duplicated without difficulty.

The temperature of 27°C is chosen because that is very near the average of the temperatures at which doughs are fermented in commercial practice. The proofing temperature, usually around 32.5°C is varied widely in practice, and continues at that temperature but a short time compared to the total fermentation time. Ten grams are chosen for a sample because the reducing sugars produced give a

convenient weight of Cu_2O on the asbestos mat. The 200 cc final volume is selected because it conveniently fills two 100. cc centrifuge tubes which balance each other. However, no error is introduced by a change of final volume to a 250, 300 cc or 500 cc flask, and the latter is sometimes desirable when working with a sample of unusually high diastatic power, such as a malt flour.

The application of this procedure to a determination of malt preparations for use in panary fermentation require some special considerations, and will be left for discussion further on in this paper.

Effect of Time and Temperature on Activity of Flour Diastase. 4. Temperature. The factors of greatest importance in the limitation of enzymatic activity have been shown by many investigators to be those of temperature, hydrogen or hydroxyl-ion concentration, and of time. Of these, the temperature factor will be considered first because it is by far the most significant in determining the production of maltose by the action of diastase under the conditions obtaining in a bread dough. Because of the very high rate of autolytic diastasis in these flour-water suspensions at temperatures near the range of optimum temperatures it was difficult to prevent large errors in the results between 50.° and 70.°C. Slight variations in temperature for the first few minutes of digestion vitiated the value of the determination. Preliminary experiments in connection with temperature control had shown that a few minutes heating of the dry flour in flasks in the water bath had no effect on the diastatic activity providing the temperature was not above 65°C. Prolonged heating, however, produces a very slow reaction due no doubt to the partial vaporization of the moisture in the flour and its condensation on the sides of the flask with consequent wetting of some of the flour. After an hour of heating at 70°C a sample of flour was found to increase slightly in reducing sugars and to decrease in diastatic power.

The procedure followed in this series was to first bring the water thermostat up to the temperature desired. The 10. gram samples of flour, in loosely stoppered erlenmeyer flasks, were placed in this bath about ten minutes before the water was added. Exactly 100. cc of distilled water in 150 cc. erlenmeyer flasks, were heated to the desired temperature in the water bath and poured quickly on to the flour sample. A sensitve 100°C thermometer was placed in the flask before adding the water. The temperatures resulting from the mixing of flour and water were usually a few tenths of a degree below that desired. They were quickly corrected by rapidly rotating the flask over the hot spot of a wire gauze heated by a bunsen flame, and the flask then replaced in the water bath. At the end of the desired time the flask was removed from the bath, transferred to a 200 cc volumetric

flask, clarified, cooled when necessary, diluted to volume, centrifuged, and the reducing sugars determined as described above. Figure 1 shows graphically the nearly autocatalytic nature of the temperature curve for one hour digestion. Three hour digestions gave a curve of the same kind. The values from which the curves are plotted are expressed in Table XIV as grams of maltose produced by the autolytic diastasis in 10. grams of flour. These values are obtained from the weights of Cu_2O corresponding to the 50. cc aliquots of the digestion solution. The blank determination, representing the natural reducing value of the extracted flour-sugars, in terms of Cu_2O, was subtrated from the total weight of Cu_2O as obtained from the auto-diastasis. The difference gave the cuprous oxide equivalent to the maltose produced by diastasis alone. The corresponding weight of maltose found from the Munson-Walker table was multiplied by the necessary aliquot number to obtain the value per ten grams (total) of sample. Each value is the average of at least three reducing sugar determinations for that time and temperature. The maltose values are calculated as "diastatic" maltose, in grams.

TABLE XIV.

The relation of temperature to the activity of wheat flour diastase.

10. grams flour No. 1009 used for each sample.

Temperature Degrees Centigrade	Weight of Maltose from Diastasis of 10. grams Flour. 1 Hour Digestion Grams
0.	.0386
27.	.2118
25.	.3238
55.	1.1378
60.	2.1396
63.5	2.9067
65.	1.6408
67.	.7698
70.	.4016
75-76	.0922
*82-83	.0244

*Gelatinized.

The maximum production of maltose was 29% at 63.5°C. But this temperature (146.3°F) is probably never reached in a dough during a normal fermentation, and only for a few minutes while baking.

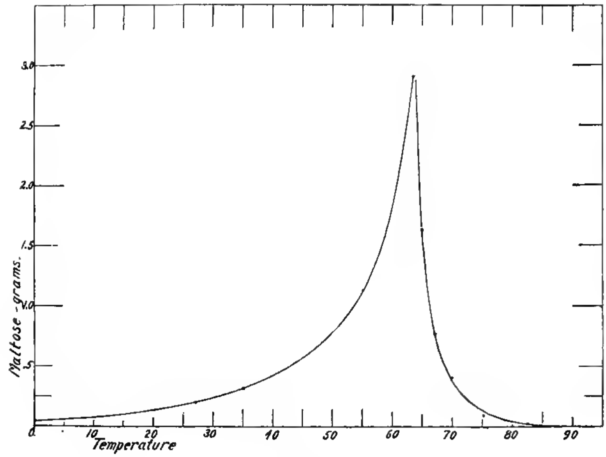

FIGURE 1.

The effect of temperature on the activity of wheat flour diastase.

5. **Time.** The relationships between activity, temperature, and time, can be conveniently considered together. Since the rate of diastasis is so enormously increased at higher temperatures, the curves for the production of maltose with time must show a corresponding increased initial rate at higher temperatures. Consequently the time curves for several temperatures were determined and are plotted in Figure 2. The data corresponding to these curves is tabulated in Table XV in groups of results, one set for each curve. To simplify tabulation the total values per 10. gram sample are calculated from the actual weighings as described for the values in Table XIV. The last set of data in Table XV is obtained on a different flour (our laboratory sample No. 1) and is also included in Figure 2 to show the similarity of the results for a flour of lower diastatic power.

TABLE XV.

The variation of diastatic activity in wheat flour with time.

Flour Number	Temp.	Grams of Maltose Produced by Diastase in 10. grams of Flour — Time in Minutes: 30	60	120	180	240	300	360
1009	0.°C.	.....	.0383	.....	.0702	.....	.....	.....
1009	27.°C.	.....	.2114	.2942	.3578	.3950	.4420	.4650
1009	35.°C.	.2334	.3235	.4497	.5181	.5832	.....	.6230
1009	55.°C.	.5608	1.0380	1.2464	1.3862	1.4916	1,4820	.....
1009	64-65.°C.	.....	1.5402	3.0796	2.6718	.....	.....	.....
1009	69-70.°C.	.....	.3801	.8225	.....	.....	.....	.....
1	27.°C.	.0725	.0963	.1313	.1423	.1632	.1741	.1870

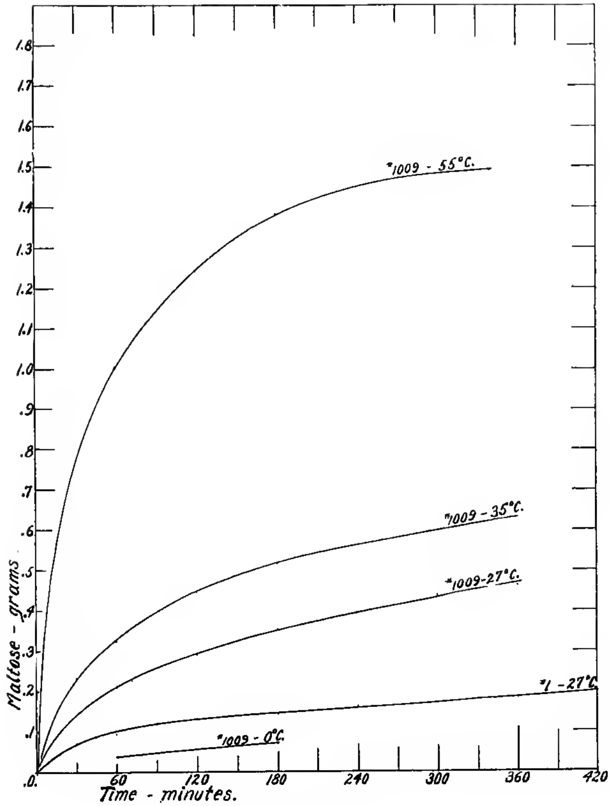

FIGURE 2.

The change of activity of wheat flour diastase with time, at different temperatures.

The curves drawn from the data in Table XV resemble very closely those which Collatz and Bailey obtained for the increase of conductivity in flour extracts due to the action of the enzyme phytase on the phytin. It would appear probable that these two enzymes exhibit a parallel activity for a given flour at any particular temperature.

The rate of maltose production has reached practically a constant value, for ordinary fermentation temperatures, between two and three hours diastasis. In the case of Flour No. 1, Table XV, the graph for maltose production with time is nearly a straight line from two to twenty-four hours.

6. **Acidity.** The diastases of many biologically different materials of both plant and animal origin, have been investigated and reported in the literature. The temperature optima vary considerably for different samples, no doubt due to adaptations to environment. The agreement, however, of the optima for hydrogen ion concentrations of most of the diastases reported seems to point to a general relationship of activity. Because of the close relationship between the barley and wheat grains the diastase of wheat would be expected to show an optimum activity at a pH of 4.7 to 5.0, corresponding to that for the diastase of malted barley reported by Sherman and Walker, and Sherman, Thomas and Baldwin.

TABLE XVI.

The influence of hydrogen ion concentration on the activity of wheat flour diastase.

10. gram samples. Flour No. 1009. 1. hour digestion @ 27 C.

Acid or Alkali Added	pH After 1 Hour Digestion	Weight of Cu_2O in Milligrams: Weight per 50. cc Aliquot	Weight of Cu_2O in Milligrams: Weight per 10 g. Flour	Corresponding Weight of Maltose in Milligrams per 10 g. Flour
25. cc N/10 HCl	1.946	15.7	62.8	+.
10. cc N/10 HCl	2.775	27.5	110.0	34.8
15. cc N/25 HCl	3.522	78.4	313.6	196.1
10. cc N/25 HCl	4.006	109.6	438.4	295.1
10. cc N/25 HCl	4.034	111.8	447.2	302.2
7.5 cc N/25 HCl	4.399	115.4	361.6	313.5
5.0 cc N/25 MCl	4.808	116.5	465.9	316.8
3.0 cc N/25 HCl	5.112	111.3	445.2	300.6
2.5 cc N/25 HCl	5.195	112.6	450.4	304.7
0.0	5.691	83.7	334.8	213.4
0.0	5.742	83.8	335.4	
2.5 cc N/25 NaOH	6.352	45.1	180.4	90.7
5.0 cc N/25 NaOH	7.023	34.7	138.8	57.4
7.5 cc N/25 NaOH	7.527	25.2	100.8	27.4
10.0 cc N/25 NaOH	9.067	19.5	78.0	9.9±.1
5.0 cc N/25 NaOH	9.134	16.5	66.0	+.
6.0 cc N/10 NaOH	9.968	16.0	64.0	+.
Blank..............	......	14.1	56.4	

Figure 3 is a graphical representation of the data in Table XVI and shows the production of maltose by diastatic enzymes with varying concentrations of hydrogen and hydroxyl-ions, in terms of pH. To obtain the data from which Table XVI is compiled, pairs of samples were run simultaneously. The regular procedure described

above was used for each sample in which the reducing sugars were to be determined, acid or alkali being added as shown in the table at the beginning of the digestion to produce the desired pH. The other, or check sample was treated in exactly the same manner up to the end of the digestion period, when instead of clarifying, the suspension was thoroughly shaken up, poured directly into centrifuge cups, centrifuged, and the supernatant liquid at once subjected to pH measurements. In this manner the resulting pH at the end of the one hour digestion was measured for each sample. For convenience in drawing the curves in Figure 3, the weights of Cu_2O equivalent to the diastatic activity were used to prepare the graph (Figure 3), rather than the corresponding weights of maltose. This was because the lower maltose values were too small to be accurately determined from the maltose tables. However, the results are expressed in milligrams of maltose actually produced by the diastase in ten grams of flour. The calculation of results in this manner have been described above.

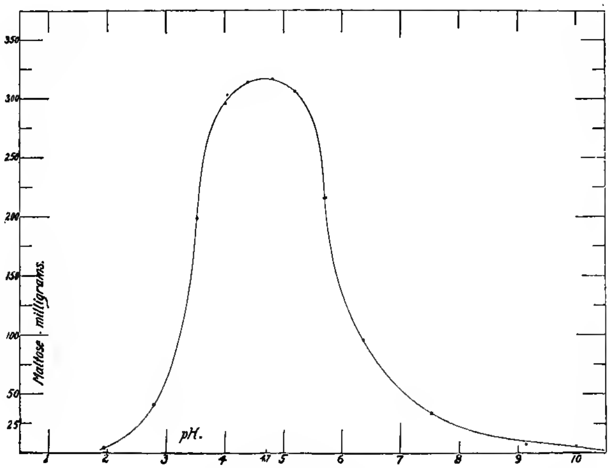

FIGURE 3.

The relationship between the activity of wheat flour diastase and the pH of the medium.

The diastatic activity of wheat exhibits practically the same maximum pH as that found for barley malt. This maximum occurs at a pH of 4.7 to 4.8, with a broader range of maxima between 4.0 and 5.3.

The rapid decrease in the production of maltose in the ranges of pH from 5. to 6.5 may be of particular significance in the fermentation of different grades of flour. It may also account for some of the differences in baking strength which become apparent with aging of the flour.

The hydrogen-ion concentration of the normal bread dough when mixed is very approximately that of the flour, usually slightly higher, and in the case of the flours here used varies from a pH of 6.15 to pH 5.6. The optimum pH for diastatic activity is never reached in the short fermentation, straight dough process, for a normal dough, and not often in sponge doughs. Even in the latter case such a high acidity is obtained only after six or more hours of fermentation, at which time the diastase has suffered greatly in loss of activity due to changes of hydration of the colloidal proteins. As the fermentation advances the hydrogen-ion concentration slowly increases, until an apparent miximum is reached somewhere around a pH 5.4 to 5.2, when the dough is ready for the oven. This point may well be that condition in which the CO_2 produced by the zymase of the yeast cells has saturated the dough. The effect of this increase of acidity may then be regarded as reciprocal, first the increasing hydrogen-ion concentration from a pH of 6. to pH 5. has the effect of nearly doubling the maltose produced per unit of time. The maltose in turn becomes immediately available as food for the yeast cells, which thereby are enabled to renew their fermentation activity and increase the rate at which the CO_2 is produced for the aeration of the dough. This increasing activity is further augmented by a temperature increased during proofing of the panned loaves; this furnishes part of the explanation for the rapid rise of the loaf in the pan just before baking. The yeast activity is likewise stimulated by the same increase in temperature and acidity. That it is the function of the diastatic enzymes present, and not of the sugar originally added to the dough, which largely controls the proofing action, is shown further on in connection with experiments on the diastatic action in actual doughs.

We should not lose sight of another important factor in the latter stages of fermentation and proofing of the dough. That is the softening effect of the proteoclastic agents on the gluten. The gluten suffers more or less rapid proteolysis: becomes softer and less elastic. Thus the increasing rate of carbon dioxide production is able to "raise" the dough easier and more quickly.

Buffer Action. The buffer effect of different flours must likewise be taken into consideration in this connection. The highly refined flours, such as patents, generally show a lower buffer value than the less highly refined, or clear flours. Therefore, the maximum acidity

should be reached more quickly during the fermentation of a patent flour, resulting in a more rapidly increasing rate of fermentation and consequently a better aeration. On the other hand the better quality of gluten in these grades of flour requires more time for its proper "ripening," is more tenacious and elastic, and so requires a greater fermentation activity to overcome its tightness. Also, its greater gas retaining capacity helps to make up a combination of strength characteristics which taken altogether, form the desirable qualities for the production of a good loaf of bread.

The flours of poorer grade, such as high percentage straights and the clears, though made from the same wheat, usually show a higher buffer value. The natural effect of their buffer salts is therefore to require a greater amount of acid formed by fermentation before the proper pH of the resulting dough is reached for maximum diastatic activity. It may be that the same concentration of hydrogen-ions, namely 10^{-5} is not reached in the doughs from low grade flours. The decreasing quality of the glutens, however, in the lower grades of flours, is oftener the limiting factor because of their very poor gas retaining capacity, and they are able to show but little improvement by the addition of acids and diastatic enzymes. Thus before the inter-relationships of these three factors, gluten strength, proteoclastic activity, and diastatic activity can be more accurately explained, it becomes necessary for further study on each one of them.

The above consideration of buffer values of flour and hydrogen-ion concentrations in the dough demonstrate the desirability of determining these values for each of the flour samples used for this investigation. The results of such a set of determinations are given in part here, and afford a confirmation of those of Bailey and Peterson (1921). The buffer values of the fourteen flours were determined by making water suspensions of each sample, of 1 : 5 concentrations, as recommended by Bailey and Peterson. These were kept at 25.°C and shaken at intervals for one hour. The suspensions were then centrifuged without filtering, and to 25. cc aliquots of the centrifugate were added varying amounts of .02 Normal HCl or $NaOH$. The resulting pH was determined at once in Bailey electrodes by potentiometer measurements. For the sake of brevity only one of the fourteen sets of readings is included in Table XVII. Figure 4 includes the graphs of three sets of values of widely different grade. The addition of .02N acid was carried up to 40. cc to better show the differences in the shapes of the curves.

TABLE XVII.

The Buffer Value of Flour Sample No. 1001.

Acid or Alkali Added to 25 cc's of 1.5 Flour Extract	pH.
12.5 cc. .02N HC1	2.519
10.0 cc .02N HC1	2.654
7.5 cc .02N HC1	2.925
5.0 cc .02N HC1	3.388
2.5 cc .02N HC1	4.150
0.0 cc	5.816
2.5 cc .02N NaOH	7.371
5.0 cc .02N NaOH	9.045
7.5 cc .02N NaOH	9.775
10.0 cc .02N NaOH	10.253
12.5 cc .02N NaOH	10.617

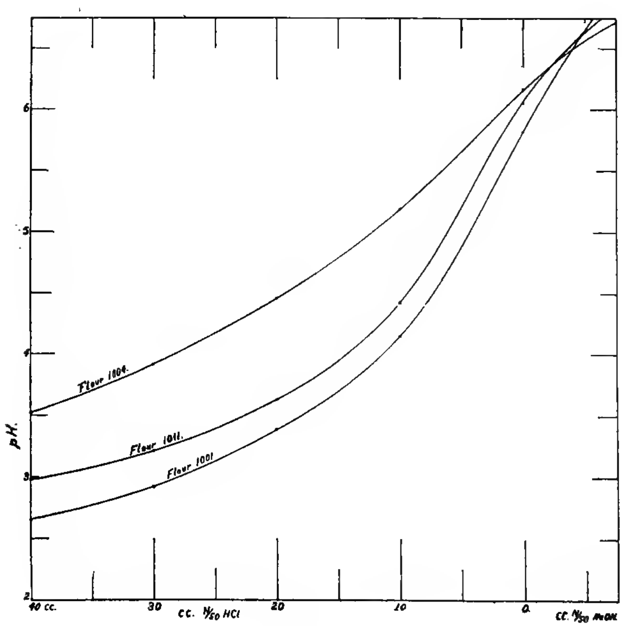

Figure 4.

The buffer curves of three flour of widely different baking value, acid range.

Because of the large number of buffer values falling within rather narrow limits, and the crossing of the curves on the alkaline side, the complete curves cannot all be drawn in.

Figure V affords a comparative idea of the buffer values in the acid range, for each of the flours used. The height of the columns represent the relative change in pH resulting from the addition of 20. cc of .02N HCl to 100 cc of the centrifuged 1 : 5 flour-water extract. The arrangement of flours is in the order of their baking value. For convenience the series of fourteen samples have been arbitrarily divided into three classes. The columns in black represent the group of flours which would ordinarily be considered by the baker as those possessing good baking qualities. The crossed lines indicate those flours of rather poor quality, but which can nevertheless be used for bread, and especially so when blended with stronger flours. The columns in diagonal lines represent those flours which are of such poor quality and low baking value that they could not be used for marketable bread in this country. While flour No. 1013 might be included in the second group because of its relative strength, its poor color would make it of doubtful value for blending.

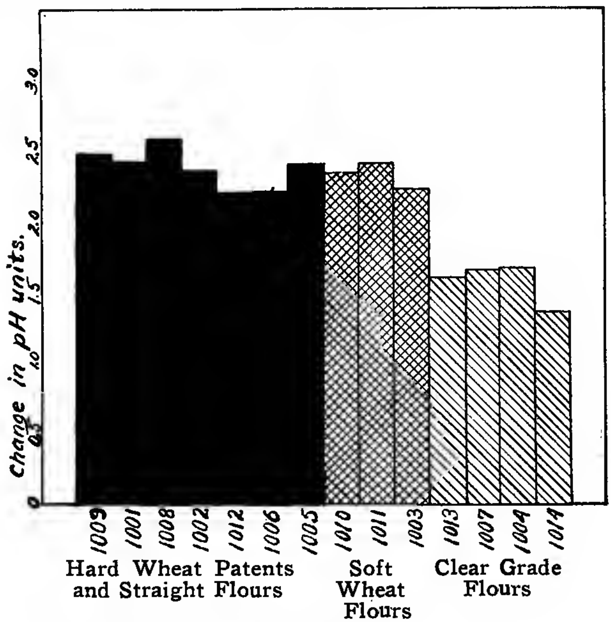

Figure 5

The relative change in pH produced by addition of 20 cc HCl to 100 cc 1:5 flour-water suspensions. Flours arranged in the order of their baking value.

There is no apparent relationship shown by the values for buffer action and the corresponding diastatic activity of the flour as given in Table XVIII. This could only be shown by a set of determinations of both pH and diastatic activity in the actual dough during fermentation. The changes of pH with progressive fermentation of the doughs made from this set of flours are given in another report. Work is now in progress in several quarters on the development of a special form of electrode for the determination of hydrogen-ion concentration in doughs. Experiments in the laboratories of one of the larger American bakeries have demonstrated the commercial application of such electrometric measurements for the control of dough fermentations.

Relative Diastatic Powers of Flour Samples. The results on determinations of the diastatic powers of each of the fourteen flours are given in Table XVIII.

The regular procedure described herein was used, namely, one hour's autolysis of a 1:10 flour-water suspension @ 27° centrigrade.

TABLE XVIII.

Comparative measurements of diastatic power on fourteen samples of flour.

Flour Sample No.	Active Sample Weighed Cu_2O per 2.5 g. Flour Grams	Inactive Sample Weighed Cu_2O per 2.5 g. Flour Grams	Diastatic Power Maltose by Diastase in 10. g. Flour Milligrams
1001	.1031	.0223	248.2
1002	.0762	.0148	186.5
1003	.0226	.0091	34.8
1004	.0638	.0155	145.0
1005	.0626	.0185	131.9
1006	.0513	.0154	105.7
1007	.0563	.0145	123.6
1008	.1146	.0162	304.1
1009	.0833	.0140	211.8
1010	.0474	.0157	92.6
1011	.0254	.0065	51.7
1012	.0529	.0085	132.9
1013	.0523	.0080	132.6
1014	.0553	.0161	116.2

The relation of the respective diastatic powers of these flours to their baking strength can be more easily shown in Figure 6, where the arrangement of flours is according to baking strength, the height of column representing the milligrams of maltose produced in one hour by 10. grams of flour. Each value is the average of three or more determinations for each flour. With the exception of the very low grade flours these samples show diastatic powers which are a fairly

good index of their general fermentation characteristics. That is, the behavior of the dough in the latter stages of fermentation, and the "spring" in the oven, show that the diastatic power of the flour as measured by the method here employed does furnish an indication of the general strength characteristics of that flour, especially with regard to volume and texture. The quality of the gluten, however, must be sufficiently high to conserve the value of the diastatic action and make a good loaf possible. It would be necessary to study many more samples of flour before extending such an observation to the status of a general conclusion.

From these samples of flour, differing so widely in type and grade, no conclusions can be drawn as to the relationship between diastatic activity of a flour and climatic factors in the growth of the wheat. Only flours produced by a uniform milling practice from wheats typical of different growing regions could be used to furnish information of such a nature.

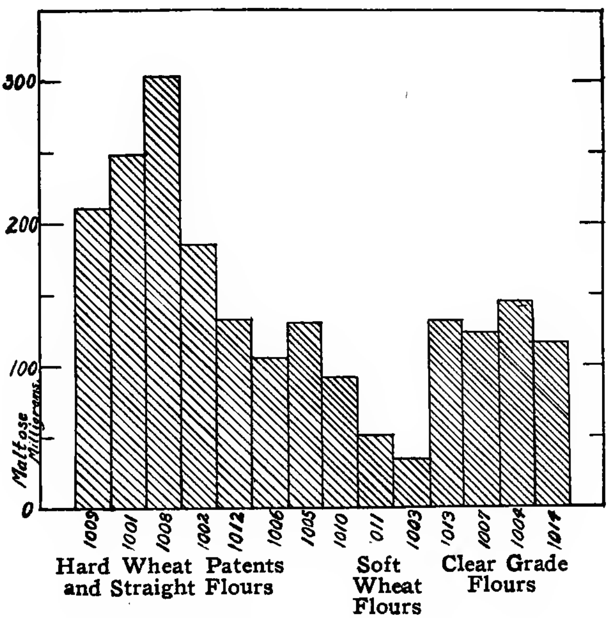

Figure 6

Relative diastatic powers of fourteen flours, in order of their baking value.

Diastatic Activity During Fermentation of the Dough. The opinions of several of the earlier investigators concerning the significance of diastatic enzymes in a dough during the latter stages of fermentation have been reviewed in the historical part of this paper.

The early English practice, revived during the late war, of fermenting doughs without added sugars would throw the burden of sugar production entirely upon the diastase of the flour unless other diastatic material, such as malt, were added. The production of carbon dioxide by the zymase of the yeast cells must continue with some regularity throughout the frementation period in order that the dough mass may be properly aerated.

The increased enzymatic activity during the forty-five to sixty minutes in which the dough is allowed to rise in the pan before baking is of especial importance. **It is this final period of aeration which determines to a large extent the texture and "lightness" of the loaf.** The gluten, however, must have the requisite quality. The proofing period is usually carried out at a temperature of eight to ten degrees higher than that of the fermentation up to this point. It is observed from Figures 1 and 2 that **this increase in temperature from 27° to 35°C increases the diastatic activity nearly 30 percent.** In actual baking practice, however, the rise of temperature in the interior of the proofing loaf is very slow and a temperature of 33 to 35°C is probably not reached until near the end of the proofing period.

If the yeast cell is to increase its zymase activity during proofing in order to provide the carbon dioxide necessary to properly raise the dough and shape the loaf, there obviously must be a sufficient supply of available sugar. If the present commercial practice of straight dough fermentation there is rarely a sufficient supply of sugar added at the time of mixing to carry the yeast activity throughout the fermentation. The diastase of the flour, if present in sufficient quantity, must carry a part of the load. The addition of a fresh supply of sugar by mixing in with the dough when ready for proofing is not practical for several reasons, and a too large supply at the time of mixing the dough often stimulates the yeast activity far beyond that desired. **The yeast must then depend largely upon the sugars produced by the diastase for its fermentation activity during the proofing.** This sugar, in the form of maltose, is readily split down to dextrose by the maltose of the yeast, and **so is immediately available as substrate for the yeast zymase.**

We might also expect that there would be a slight difference shown in the rate of carbon dioxide production between the action of yeast fed on sucrose added at mixing, and on maltose produced by diastase in the dough. The latter may be regarded as a steady rate of maltose

formation within the colloidal medium **immediately surrounding** the yeast cells. The distance the maltose must diffuse to reach the active yeast surface is thus at a minimum. On the other hand the utilization of sugars added to the dough must decrease in rate as the diffusion distance from the active yeast interface increases. This would further lead to the opinion that in theory the substitution of the requisite diastatic enzymes for a part of the sugar usually added to the dough at mixing should produce a better and more uinform fermentation, and in general a better loaf. Some sugar of course should be present at the beginning of the fermentation to stimulate the yeast activity and supply food for the early fermentation, unless the whole fermentation period be lengthened accordingly. After that time the diastase should have reached nearly a constant rate of maltose production and should, therefore, be able to supply the necessary sugar in a **more available** form.

In practice, however, the addition of considerable amounts of diastatic malt preparations, e. g., malt flours or malt extracts, is complicated by the excessive activity of the proteoclastic enzymes which they usually contain. These malt preparations likewise often contain pigmented material which is not destroyed during fermentation. In fact some of the pigmented particles appear to be so affected by fermentation that they are made soluble and tend to diffuse into the dough, darkening the color of the finished product. Here again the importance of the proteoclastic enzymes and the necessity for their measurement and control, often overshadow the practical application of diastatic malt products in the bakery.

It appeared highly desirable, therefore, to make some measurements of sugar content and diastatic activity in doughs during the normal fermentation. Regular test doughs were accordingly mixed, omitting either the yeast, or sugar, or both, depending upon which type of resultant sugar content was to be measured. The lengthy method of extracting the sugars from these doughs with alcohol, followed by evaporation, dilution, clarification, etc., is hardly suited to the rapid determination of total and reducing sugars in several doughs at short intervals. The procedure which had been previously used for the measurement of diastatic activity in doughs suggested itself as a possible basis for a satisfactory method. Several trials at 30 minute intervals during the fermentation of a dough without yeast, gave results which when plotted in terms of sugars produced per unit of time, showed a smooth curve corresponding roughly to the enzyme curves of Figure 2. The curves for a number of flours indicate that the method will at least give comparative results for each type of sugar content and for each flour.

The procedure finally used for these determinations was as follows: A two-loaf dough was mixed as for a baking test in the manner previously described. This was taken from the mixer and divided into two equal batches, one of which was fermented and baked, and used as a control dough, the other being handled in the same manner except that from it samples were taken at desired intervals. From these samples two ten gram portions were weighed off, one taken immediately for sugar analysis, and the other used for the determination of total solids. The sample to be analyzed for sugars was at once shaken up in a 500 cc bottle, with about 100 cc of distilled water to which two or three drops of 50% NaOH had been added, until the gluten had been dispersed and the sugars dissolved out. This dough suspension was poured into a 300 cc volumetric flask, clarified and treated as described above in the regular procedure. In some cases a 50. cc aliquot of the clear centrifuge was taken for hydrolysis and determination of total sugars by the A. O. A. C. method. The results for the total sugars while substantiating the conclusions to be drawn from those of the reducing sugars, do not add materially to the information desired, and are not included here, as they were not obtained in complete sets for all samples.

Many determinations were made in this laboratory on the effects of diastatic action during the fermentation of doughs. Different kinds of diastatic malt preparations, in varying amounts were used in the doughs, with and without other sugars, and with and without yeast. The results are included in a separate report on flour strength as influenced by the addition of diastatic ferments. (Collatz, 1922). Only a few examples have been selected from this available data to show the role which diastase plays in panary fermentation. These are designated by Roman numerals from I to VIII, corresponding to the curves in Figures 7-10. The experiments with these doughs were made in pairs, one dough of each pair was made up with the usual amount of yeast, the other without yeast. In this manner the relation of sugar produced by the diastase to that required by the yeast fermentation under the same conditions to temperature is clearly shown. Dough I was the regular baking test dough, containing 3.0% of sucrose and 2.5% yeast. The resulting loaf was above the average, though slightly overproofed. The texture was fair, the volume large (2110. cc), and the color good, but the grain was a bit too open because of overproof. Dough II was the same as No. I except that the yeast had been omitted. Dough III was mixed without sugar, but otherwise the same formula as Dough I. The fermentation time was necessarily longer. The resulting loaf was of very good quality, of very fine grain, good texture and an even but strong break. The color of crust

was fair, but considerably lighter than that of loaf I, as would be expected from the lack of sugar. The volume likewise was less, being only 1870. cc. Dough IV was the same as No. III, omitting the yeast. The supply of flour No. 1009 was exhausted at this point and another sample was obtained from the same mill. This second sample, designated as No. 1009A was somewhat inferior in strength and color, but the behavior of its diastatic enzymes were practically the same as those of No. 1009. Diastatic activity and reducing sugar determinations for doughs mixed with and without sucrose showed practically the same curves as those for doughs Nos. 1 to IV. Flour No. 1009A was, therefore, used for doughs V to VIII. Dough V was mixed with 2.% of a malt flour as a source of added diastatic enzymes, but no other sugar was added. Although the amount of this particular malt flour was somewhat in excess, as indicated by the grayish color of the crumb and the slight coarsening of the grain, the effect of the added enzyme is clearly shown in curve V. The volume of the loaf was increased 12% over that of a standard dough containing sucrose but no added malt diastase. The further discussion of this phase of the question of added diastatic enzymes is considered in detail in the second report. (Collatz, 1922). Dough VI was mixed with the 2.% of malt flour but contained no yeast. Dough VII: The total amount of maltose produced in dough V by diastatic action of combined wheat flour and malt flour was calculated back into terms of anhydrous maltose per unit weight of flour. This percentage of anhydrous c.p. maltose was then added to Dough VII, without other sugars or diastatic products. The resulting loaf bore the same relation to the standard loaf as in the case of Doughs Nos. I and II, namely, a finer grain, good texture, lighter color of crust, and slightly smaller volume. Table XIX is furnished here as an example of the data obtained for each dough, and comprises the basis from which the points were plotted in Curve III, Figure 8. To save space and to avoid multiplicity of headings the data for the complete set of curves, Figures 8, 9, 10 and 11, are abbreviated and grouped in Table XX.

TABLE XIX.

Reducing Sugars and Diastatic Activity During the Fermentation of a Dough. Mixed Without Sugar. Curve III.

Sampled At	Total Time	Temperature	Weight of Dough	Weight of Total Solids per 10. g. Dough	Weight of Cu_2O per 10. g. Dough	Reducing Sugars as Maltose per 10. g. Dough
	Minutes	Centigrade	Grams	Grams	Milligrams	Milligrams
Mixing	0.0	27.°	530.0	5.5720	207.0	156.0
1. hour	60.0	27.5°	.....	.....	274.4	209.5
1st Punch	177.0	27.5°	526.0	5.5222	269.4	205.5
2d Punch	252.0	27.7°	522.0	5.5086	197.4	148.3
To Bench	297.0	28.5°	519.0	5.5261	168.0	125.2
To Proof	312.0	.....	.....	.....	.....	.....
To Oven	359.0	36.0°	.....	5.5476	116.8	84.6
Out Oven	377.0	(226.5°)	.....	.....	187.6	140.5

TABLE XX.

Weights of Reducing Sugars Calculated as Milligrams of Maltose in Ten Grams of Dough at Different Stages of Fermentation.

Sampled At:	Straight Dough 2.5% Sucrose I. With Yeast	II. Without Yeast	Straight Dough No Sugar III. With Yeast	IV. Without Yeast	No Sugar 2% Malt Flour V. With Yeast	VI. Without Yeast	Straight Dough 1.83% Maltose VII. With Yeast	VIII. Without Yeast
Mixing	481.9	99.9	156.0	125.7	231.6	184.6	231.0	200.9
1 Hour	456.6	151.0	209.5	159.4	307.9	235.7	288.7	230.8
First Punch	395.8	178.2	205.5	204.1	278.2	286.5	279.3	288.9
Second Punch	274.1	240.2	148.3	222.0	308.6	326.1	261.3	306.0
To Bench	222.6	260.2	125.2	233.6	.....	.....	254.2	312.4
To Proof	.....	.....	.....	.....	333.8	364.3	.....	.....
To Oven	186.8	256.6	84.6	245.3	347.8	365.0	211.9	320.6
Out of Oven	209.4	624.1	140.5	.....	258.7	.....	272.8	.....

The conclusions to be drawn from this set of results in Table XX are verified by considerable additional data obtained on other flours, as included in the second report. Curve I, Figure 7, shows the rapid consumption of reducing sugar by the yeast zymase in the production of carbon dioxide and alcohol. The fall of reducing sugar content slows up about the time the dough goes to the proof-cabinet, indicating that the increased diastasis due to increased temperature is making itself felt. This latter increase is shown nicely by Curve II. The wide difference between the reducing sugars present in the two doughs

is of significance, considering that in Dough I the sugar was added as non-reducing sugar (sucrose). This must, therefore, be ascribed in part, if not entirely, to the rapid hydrolysis of sucrose by the invertase of the yeast cells. Otherwise it would likewise appear in Curve II. In other words, the yeast cell appears to invert the sucrose faster than its zymase activity requires. This same result is observed in every case where this sort of determination has been made. In the other pairs of curves the divergence is not so great because the amount of disaccharides present are much smaller.

Curve III, Figure 8, emphasizes the fact, as indicated by Curve I, that the yeast's requirement of available sugars continues throughout fermentation, and really increases with the stimulated activity during the proofing of the loaf. In this curve the yeast zymase has practically exhausted the supply, and the volume of the baked loaf suffers in consequence, even though the flour diastase (Curve IV) is still active. Curves V and VI, Figure 9, on the other hand, illustrate an entirely different state of affairs. With a sufficient supply of diastatic power the excess of reducing sugars (above the yeast requirement) instead of falling off after three hours of fermentation as in the two previous cases, is carried along in an upward curve until it is no longer needed. The result is an increase in loaf volume. When this added diastase can be supplied without the associated undesirables of coloring matter and excessive proteoclastic action, there should be no question as to its importance in the manufacture of good bread. Curves VII and VIII, Figure 10, show much the same situation as those for I and II. The maltose, as sugar added at mixing, does not appear to sustain the fermentation in its later stages any better than sucrose. The fermentation curve, so-called, does appear to be more uniform, but whether that difference depends upon the nature of the sugars is still to be determined.

One more point remains to be noted. The sudden increase of reducing sugars during the first few minutes in the oven is shown by the dotted lines at the end of curves I, II and VII. The sudden increase of heat in the oven, **decreasing in rate from the exterior to the interior of the loaf**, during what the baker terms the "oven spring," should be expected to steadily raise the diastase up to and beyond its optimum temperature. The resulting sugars produced in the loaf must then be taken into consideration in the interpretation of carbohydrate anaylsis of bread.

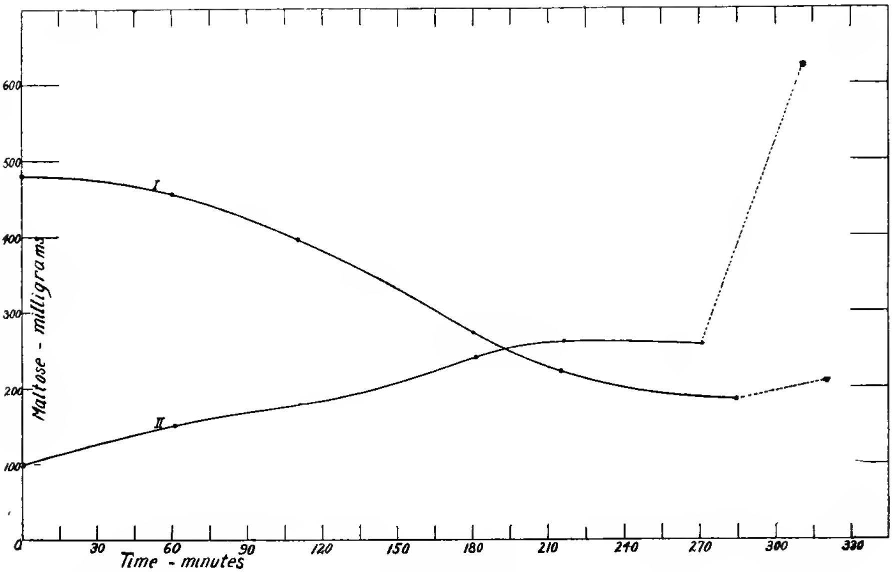

Figure 7.

Reducing Sugars Found in Doughs During Fermentation. Curve I. Straight Dough. Standard Formula. Curve II. Straight Dough. Yeast Omitted.

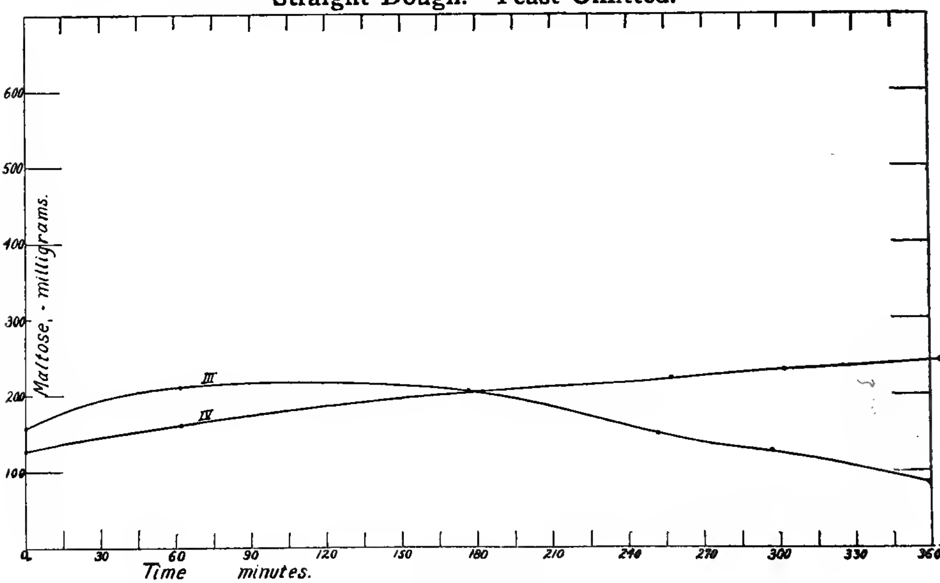

Figure 8.

Reducing Sugars Found in Doughs During Fermentation. Curve III. Straight Dough. Sugar Omitted. Curve IV. Straight Dough. Yeast and Sugar Omitted.

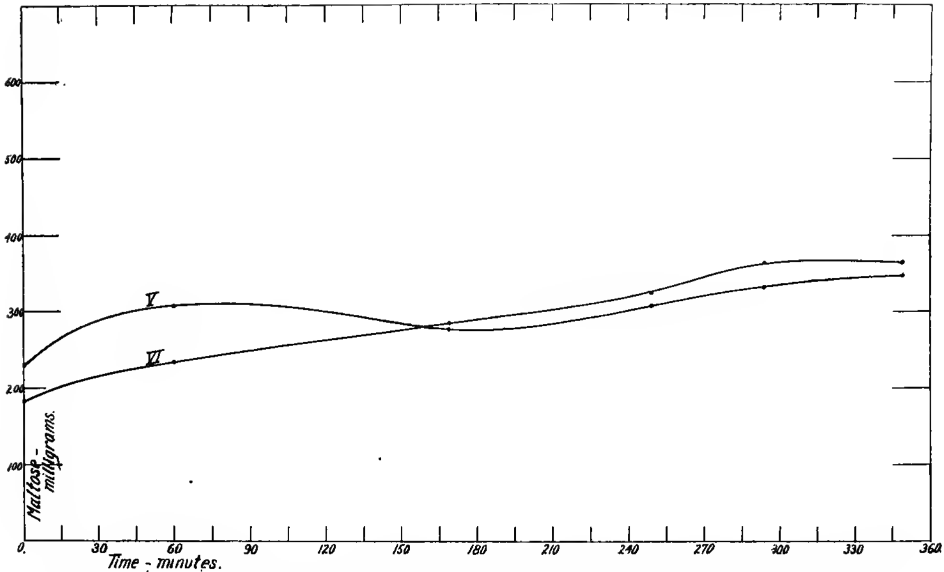

Figure 9.
Reducing Sugars Found in Doughs During Fermentation. Curve V. Straight Dough. Diastatic Malt Added. Curve VI. Straight Dough With Added Diastase, But Yeast Omitted.

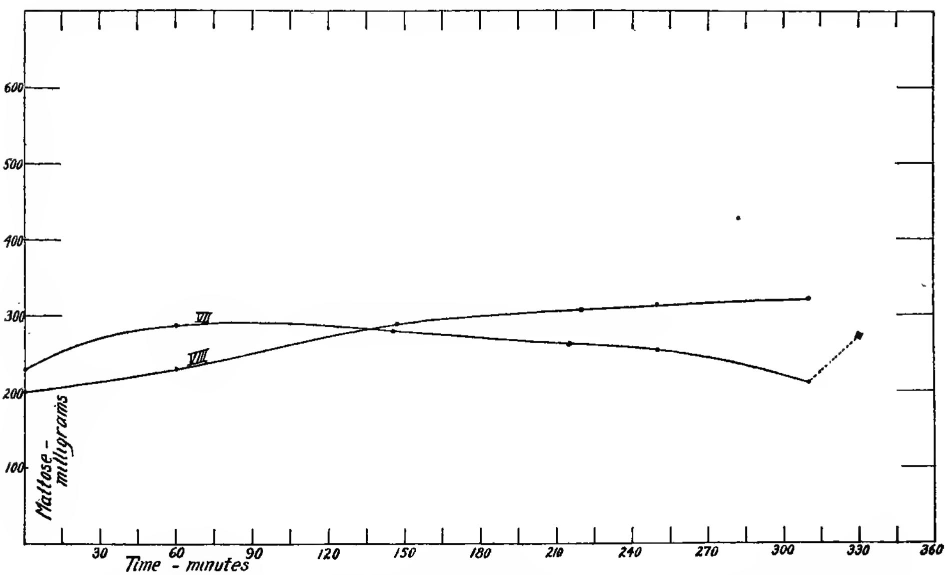

Figure 10.
Reducing Sugars Found in Doughs During Fermentation. Curve VII. Straight Dough. Maltose Substituted for Sucrose. Curve VIII. Straight Dough. Maltose Substituted, Yeast Omitted.

The Relation of Diastatic Power to Different Forms of Starch. Before the various diastatic preparations, such as barley malt flours and malt extracts, can be used intelligently in panary fermentation to supply the required diastatic power, it is necessary to have some rational basis for measurement. The diastatic power of these products should be expressed in terms of their ability to produce maltose per unit weight used, under the conditions of baking practice. It becomes necessary first to find an easily standardized substrate which will be comparable to the flour which goes into the dough. The more accurate way of determining the diastatic activity of a malt product upon the particular flour with which it is to be used offers many objections, both technically and as a basis for control and commerce. The use of wheat flour as substrate involves the determination of the diastatic power of the flour itself, the condition or resistance of the starch granules to the enzyme, the acidity and buffer value of the flour; in short, as many factors as there are flours.

The next possibility is in the selection of a standard starch as substrate, under controlled conditions of acidity and salt concentration, which will give results comparable to average bake shop conditions. Preliminary experiments have furnished some data which are given here because of their general bearing on this phase of the problem.

Five forms of starch were used in this work. Starch sample number 1, was the starch as it naturally occurs in flour sample No. 1009, with its associated diastase, and other enzymes, salts, and organic materials. Number 2 was a sample of Merck's "soluble starch according to Lintner," used in water suspension without heating. No. 3 was the same soluble starch gelatinized by boiling. Sample No. 4 was commercially prepared wheat starch. Sample No. 5 was a sample of wheat starch prepared in this laboratory as previously described.

The researches of Thatcher and Koch (1914), Thatcher and Kennedy (1920), and Sherman, et al. (1913-1921), furnish examples of the use of soluble starch as substrate for diastatic enzymes. The contribution of Swanson and Calvin (1913), showed the effect of autolytic diastasis on natural wheat starch. The differences between these two forms of substrate for diastatic activity are obvious from the published data. To determine whether the diastase in wheat flour would act more readily on added soluble starch than on the unchanged starch granules of the wheat itself, the following experiment was carried out.

One 10 gram sample of flour No. 1009 was digested with 100 cc of water for one hour at 27°C in the regular manner. To a second sample were added 10 grams of soluble starch (No. 2), and handled in the same manner. Blanks were run for both samples to determine their natural reducing values. The results are given in Table XXI,

and are the averages of three or more determinations on each sample. There is a considerable augmentation of the maltose produced by the addition of a more easily digested form of starch to the diastatic medium. The slight increase in acidity due to the addition of the more acid soluble starch might account for a part of the difference, as shown by the data in Table XXII.

TABLE XXI.

The Activity of Wheat Flour Diastase on Wheat Flour Starch and on added Soluble Starch.

Starch Used	Actual Weight of Cu_2O per 50. cc aliquot of Solution Milligrams	Difference Calculated as Maltose by Diastase in 10. g. Flour Milligrams
10. g. Starch No. 1 (Wh't flour No. 1009 only)	82.3	208.2
10. g. Starch No. 1 (Blank)	14.1	
10. g. Starch No. 1+10. g. Starch No. 2	114.9	275.0
10. g. Starch No. 1+10. g. Starch No. 2 (Blank)	25.6	

The effect of several different forms of starch on the activity of a diastatic malt extract was next tried. There was at hand for this work a fresh sample of a commercial diastatic malt syrup which had just been analyzed for total solids, ash, and proteins, total reducing sugars, and the Lintner value of which was determined as 44.4.

The starches used as substrate were weighed out in 10 gram samples into 300 cc erlenmeyer flasks and shaken up with 100 cc of water. To these there were added 10 cc of a freshly prepared 5% solution of the malt extract. These were allowed to digest one hour at 27°C, and then treated in the regular manner. Because of the high acidities of some of the digestion samples they were repeated, using a solution of K_2HPO_4 and KH_2PO_4 as buffer salts to control the pH. For convenience the two sets of experiments are grouped together in table XXII to show the amounts of maltose produced by the same amounts of diastase under different conditions.

An inspection of the pH values in Table XXII, considered in relation to the graph in Figure 3, shows that the points obtained lie for the most part on either side of the optimum range, and a slight change toward neutrality should result in a considerable increase of maltose production. These values for the pH are likewise rather far from those which normally obtain in a dough, with the exception of Starch No. 2. Consequently the maltose produced by the diastase under these conditions cannot be taken as a comparative measurement of diastatic power of the malt extract in a dough.

TABLE XXII.

Maltose produced by the same weight of Malt Diastase acting on different Starches for one hour at 27°C.

Starch used as Substrate	pH at end of Digestion	Weight of Cu_2O per 50 cc of Digestion Solution Grams	Maltose Produced by Diastase of 1 gram Malt Extract Milligrams
No. 1. (Flour 1009) Sample	5.801	.2522	247.0
No. 1. (Flour 1009) Blank		.1284	
No. 1. (Flour 1009 without added malt) (Sample)		.0823*	
No. 1. (Flour 1009 without added malt) (Blank)		.0141*	
No. 2. (Soluble Starch) Sample	5.204	.1584	200.8
No. 2. (Soluble Starch) Blank		.1272	
No. 2. (Soluble Starch + Buffers) Sample	6.847	.2183	561.4
No. 2. (Soluble Starch + Buffers) Blank		.1772	
No. 3. (Gelatinized Starch) Sample		.4119x2	2759.4
No. 3. (Gelatinized Starch) Blank		.0652x2	
No. 4. (Com'l Wheat Starch) Sample	4.108	.1492	157.8
No. 4. (Com'l Wheat Starch) Blank	1.014	.1218	
No. 4. (Commercial Wheat Starch + Buffers) Sample	6.707	.1458	136.0
No. 4. (Commercial Wheat Starch + Buffers) Blank			
No. 5 (Prepared Wheat St'ch) Sample	4.924	.1470	215.2
No. 5. (Prepared Wheat Starch) Blank		.1105	
No. 5. (Prepared Wheat Starch + Buffers) Sample	7.098	.1324	116.8
No. 5. (Prepared Wheat Starch + Buffers) Blank		.1105	

*(Values taken from Table XXI to furnish a correction for the diastatic activity of the flour No. 1009.)

On the other hand, the similarity in result between Starch No. 1 with a pH of 5.8 or less, Starch No. 2 with a pH of 5.2, and of Starch No. 5 with a pH at 4.9, would indicate the probable satisfactory solution of the problem. The results for Starch sample No. 3 confirm the statements as reviewed earlier in this report. There can then be no question as to the fallacy of reporting the diastatic power of a malt product for bread making in terms of its action on gelatinized soluble starch. It is hoped that a completed report can be made on this phase of the work at an early date.

Additional analytical data on each of the fourteen flour samples used in these experiments is compiled for reference, and is appended as Table XXIII.

TABLE XXIII.

Analytical Data on Flour Samples.

Sample No.	Moisture %	Ash %	Protein %	Wet %	Dry %	Acidity As Lactic Acid, %	pH	Baking Value
1009	11.61	.423	13.81	31.21	11.18	.20	5.981	100
1001	12.15	.405	11.34	35.21	11.09	.14	5.816	99
1008	11.70	.459	15.32	44.65	15.44	.20	6.133	97
1002	12.14	.610	13.00	41.97	13.28	.16	6.052	95
1012	12.72	.407	12.76	38.42	12.94		5.732	91
1006	11.96	.378	11.96	35.49	12.96	.135	5.777	91
1005	11.27	.431	11.04	29.15	11.15	.14	5.843	90
1010	12.48	.539	10.36	30.77	10.99	.18	5.961	83
1011	11.43	.562	10.77	29.63	10.71	.22	6.05	76
1003	13.06	.463	8.83	25.13	9.22	.11	6.044	63
1013	12.83	.543	14.83	44.55	15.63		5.596	56
1007	11.06	.637	14.12	36.58	14.18	.20	6.132	46
1004	10.58	.829	12.70	29.58	12.11	.26	6.166	35
1014	12.22	.795	14.37	39.96	14.47		5.843	32

SUMMARY

Diastatic enzymes are recognized as one of the important factors which go to make up "flour strength."

Experiments to define more clearly the action of diastase in the production of better bread are indicated by a review of the literature dealing with the different factors of flour strength.

The effects of diastatic enzymes of the wheat flour in panary fermentation, with respect to concentration, time, temperature, acidity, and diastatic power, have been determined.

Representative flour samples, numbering fourteen in all, were collected from typical wheat growing regions of North America to serve as a basis for the study of diastatic enzymes in bread making.

The baking values of the flour samples were determined by means of comparative baking tests. Other available analytical data has been tabulated along with these baking values in order to correlate as many different factors as possible with the "strength" of each flour.

Protein precipitation from cereal extracts by the tungstic acid reagent of Folin and Wu was studied in its application to the determination of diastatic power in flour. A convenient, rapid, and complete removal of proteins from solution, without filtration, is effected by the use of 3. cc of 15% sodium tungstate per 10 grams of flour, acidifying with sulfuric acid to a pH of 2, with subsequent centrifuging. Diastatic activity is completely inhibited by this treatment.

Diastatic powers of fourteen flour samples were determined by one hour autolytic digestion at 27°C. The maltose produced serves as a measure of the diastatic activity of that flour when in the form of a dough.

Maximum activity of flour diastase in a dough at any given temperature or pH is not reached at once because of insufficient water. The slower hydration of the colloidal materials compensates in some measure for the more rapid loss of activity which the diastase would suffer in water suspension, and hence the rate of maltose production in the dough is quite regular at any given temperature.

Optimum hydrogen ion concentration for flour diastase, pH of 4.7, is seldom reached during the fermentation of a normal dough. The increase of acidity during fermentation, in the range of pH 6 to 5, has the effect of considerably increasing the production of maltose toward the latter part of fermentation. The effect of hydrogen ion concentration on the activity of flour diastase is in agreement with that found by Sherman and others for the diastase of malt.

Temperature is the most important factor in the control of diastatic action in the dough. The increased temperature at proofing, along with the increased hydrogen ion concentration, combine to make the effect of diastase most significant during the later stages of fermentation. It is during this later period that the diastase produces the necessary sugars from which the yeast may complete the aeration of the dough during the proofing of the loaves.

The flour showing the greater diastatic power should show the greater strength and consequently the greater baking value, providing the relative quality and quantity of the gluten is the same.

A rational standard method is needed for measuring the diastatic materials such as are in general use in bread making. Experiments are described which demonstrate the necessity for using a standard starch substrate, and a possible method for preparing such a substrate is suggested.

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BIOGRAPHICAL.

Louys A. Rumsey was born May 7th, 1889, at Stryker, Ohio. He attended the public schools and graduated from the high school there in 1908. Entering Denison University, Granville, Ohio, in the fall of 1908, he specialized in chemistry and graduated with the B. S. degree in June, 1912. He returned to Denison the following year and received the M. S. degree in June, 1913. The thesis work was done in the department of chemistry on some reduction compounds of tin, under the direction of Professor A. M. Brumbach. Following a summer's work in the final inspection department of the Dayton Engineering Laboratories, Dayton, Ohio, he went in October, 1913, to the Iowa State College of Agriculture and Mechanic Arts as an instructor tor in the department of chemistry. He remained there as instructor in home economics and food chemistry until September, 1917, when he returned to Denison University as Assistant Professor of chemistry. He had taken work in the graduate school of Chicago University during the summer quarters of 1914 and 1916.

After a three months' training at Fort Sheridan, Illinois, during the summer of 1918 he was given a certificate of Commission as second lieutenant and sent back to teach chemistry at Denison.

He enrolled as a student in the graduate school, University of Minnesota, in September, 1921. Having been granted a fellowship by the American Institute of Baking, Minneapolis, the work on his thesis was carried out in their laboratories, under the direction of Dr. R. A. Gortner and Dr. C. H. Bailey of the University of Minnesota, Division of Agricultural Biochemistry.

In May, 1922 he presented to the faculty of the graduate school a thesis on "The diastatic enzymes of wheat flour and their relation to flour strength," in partial fulfillment of the requirements for the degree of Doctor of Philosophy. The degree was conferred June 14th, 1922 by the University of Minnesota.

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L. A. RUMSEY.